**Regulation of Bladder Cancer Invasion**

by

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## DEDICATION

To my grandmother who passed away from liver cancer (Aug. 13, 2001).

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## ABSTRACT

The most common type of bladder cancer is urothelial carcinoma (UC). Bladder cancers are categorized as either non-muscle invasive (stages Ta-T1) or muscle invasive (stages ≥T2). The majority of bladder cancers are non-muscle invasive at initial diagnosis; however, the recurrence rate for these tumors is high and a subset of them progress into T2. In this study, we aimed to determine if there is differential gene expression between T1 versus T2 bladder cancers that can help identify key regulators in bladder cancer progression and invasion. T1 and T2 bladder cancer tissues were subjected to RNA-Seq to evaluate differential mRNA expression amongst these stages. The Oncomine database was then examined to further limit potential candidates that differentiate T1 from T2. These efforts led to the identification of an extracellular matrix glycoprotein, *fibulin-3* (also known as *EFEMP1*), as being highly expressed in T2s compared to T1s. Consistent with these findings, fibulin-3 expression level correlated with the invasive ability of several bladder cancer cell lines. Specifically, fibulin-3 expression was determined using both qRT-PCR and western blotting amongst the T24, UMUC-13, UMUC-3, RT4, and 5637 bladder cancer cell lines. The most invasive cell lines, T24 and UMUC-13, demonstrated the highest fibulin-3 expression. In contrast, the least invasive cells, RT4 and 5637, demonstrated the lowest fibulin-3 expression. Genetically-engineered modulation of fibulin-3 expression in bladder cancer cell lines was directly associated with their invasive ability. Knockdown of fibulin-3 in bladder cancer cell lines decreased the incidence of muscle invasive

bladder tumors in a murine orthotopic bladder cancer model. Fibulin-3 knockdown in bladder cancer cells decreased their expression of IGFBP5 and restoring IGFBP5 rescued their invasive and migratory potential. These results indicate that fibulin-3, in part through modulating IGFBP5, serves as a pro-invasive factor in bladder cancer. These findings suggest that fibulin-3 and IGFBP5 could serve as both (1) biomarkers to identify potential muscle invasive bladder cancers and (2) promising therapeutic targets for bladder cancer.

# CHAPTER I

## Introduction to Bladder Cancer

### Epidemiology

Bladder cancer is the eighth most common cause of cancer death in men (1). It is estimated that 76,960 new cases of bladder cancer will be diagnosed and 16,390 deaths will arise in 2016 due to the disease (1). Men are 3-4 times more likely than women to get bladder cancer (1). While men have a 1 in 26 chance of developing bladder cancer in their lifetime, women have a 1 in 88 chance. However, women are often present with more advanced disease and a less favorable prognosis for as of yet unclear reasons (2). Although bladder cancer is typically considered a disease of the elderly, with the average age of diagnosis at 73, it can occur at any age and the risk of developing bladder cancer increases and patients' clinical presentation and outcomes can worsen with advancing age (3). Bladder cancer is also more common in developed countries such as North America and Western Europe as well as in Caucasians than the other races (4).

### Risk Factors

One of the key risk factors of bladder cancer is smoking, which actually accounts for half of the cases (5) and increases the chance of developing bladder cancer by 3 times compared to not smoking (6). Although the exact mechanism through which smoking induces bladder cancer is still unclear, it is believed that chemicals from the cigarettes are eliminated in the urine after

being filtered by the kidneys. This allows direct contact of the cigarette-derived chemical carcinogens with the bladder mucosa, potentially causing the onset of bladder cancer. However, despite the decrease in smoking rates, incidence of bladder cancer has remained stable (7). In addition, men are still at a higher risk of developing bladder cancer than women even amongst non-smokers, suggesting that other factors such as environmental exposures and genetic predisposition can also attribute to the development of bladder cancer.

Occupational carcinogen exposure is also an important risk factor (8). People who work in factories with paint, textiles, rubber, leather, and dyes and are exposed to the chemicals in those materials (i.e. aromatic amines) have been linked to a higher risk of bladder cancer (9). Besides occupational exposures, environmental factors such as consumption of water with high concentrations of arsenic (10) or chlorine (11) have been associated with an increased risk as well. On a related note, amount of fluid intake has been presented as another potential risk factor as the increased amount of water has the potential to dilute the excreted carcinogen and limit the exposure to the urothelium (12). Analogously, frequent urination may help eliminate the carcinogens from contact with the bladder.

Although changes can be made to limit exposure to the above risk factors, other factors such as genetic predisposition cannot be altered. Along these lines, it has been found that some families tend be more susceptible to bladder cancer than others as relatives of people with bladder cancer are at a higher risk of getting it themselves (13, 14). It is plausible that exposure to similar environmental conditions occurs within a family; however, that alone cannot account for all of the bladder cancer cases (15). Although familial bladder cancer does not occur frequently, a few reports have indicated a potential familial component (15, 16). Consistent with this possibility are studies on genetic susceptibility of bladder cancer risk, which have revealed

inherited genetic factors that increase the risk for developing bladder cancer. These genes may potentially explain the higher incidence as it relates to carcinogen exposure for these families as some of them involve enzymes that detoxify carcinogens, such as glutathione S transferase Mu1 (GSTM1) and N-acetyltransferase 2 (NAT2). It was reported that GSTM1-null genotypes have a 50% increased risk of bladder cancer (17). GSTM1 deletions also have been found in about half of the Caucasian population in the United States (18), which could help explain the higher bladder cancer incidence seen in whites. Alterations in the NAT2 alleles have likewise been reported to increase the risk of bladder cancer (19), especially in the case of cigarette smokers (20). Although these genes may not directly lead to bladder cancer, they could help facilitate the onset of bladder cancer after being exposed to different carcinogens. It supports the notion that only a small percentage of individuals develop bladder cancer compared to the many people who are exposed to the different environmental risks. Thus, a better understanding of how bladder cancer develops could be gained by considering both environmental and genetic factors.

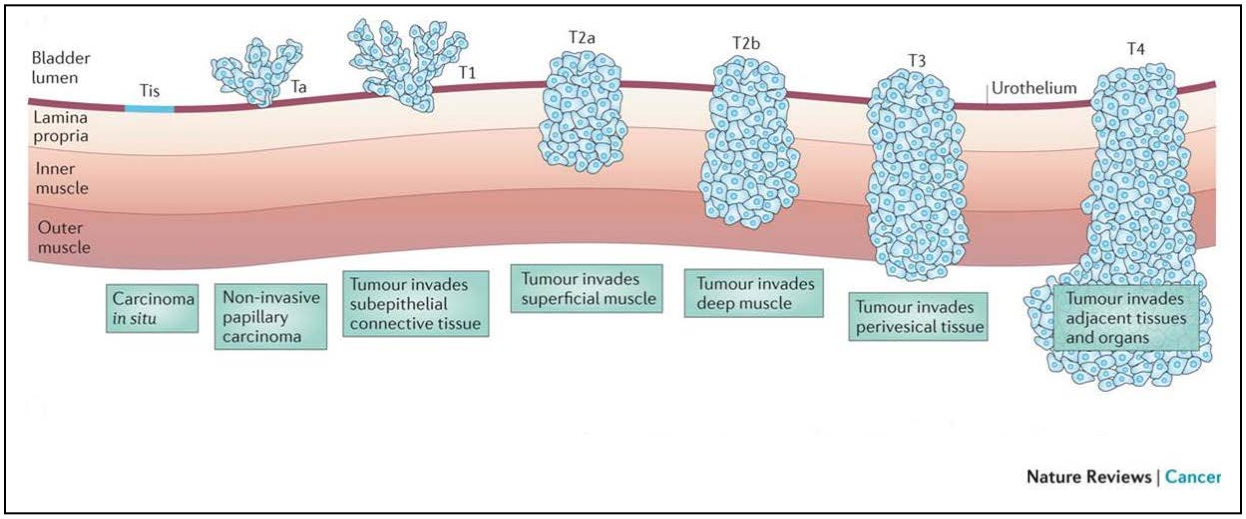


**Figure 1. Bladder cancer stage classification based on depth of tumor invasion in the urothelium and presence of metastasis.** Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, (21), copyright (2015)

**Clinical presentation/pathology**

Urothelial carcinoma (UC) also known as transitional cell carcinoma (TCC) is the most common type of bladder cancer (22). Other types of bladder cancer include squamous cell carcinoma, which actually only accounts for 2-5% of the bladder cancer cases (23) and adenocarcinomas, representing only 1% (24), however mixed histology is relatively frequent. UC arises from the urothelial cells in the bladder and can typically be categorized as either non-muscle invasive bladder cancer (NMIBC), stages Ta-T1 or muscle invasive bladder cancer (MIBC), stages ≥T2. Bladder cancer is staged based on its degree of invasion and metastasis (Fig. 1). In the majority of cases (75-85%), patients are first diagnosed when the cancer is still confined within the inner layer of the bladder, non-muscle invasive Ta-T1 (25). Ta: Non-invasive papillary carcinoma is an early cancer found only on the surface of the bladder inner lining and can be of either low or high grade. The cancer cells are typically grouped together and can be easily removed. On the other hand, Tis: Non-invasive flat carcinoma (flat carcinoma in

situ, CIS), which is also found only on the inner lining of the bladder, does not grow towards the bladder lumen. Tis are high-grade and have the propensity to invade (26). For stage T1, the tumor has grown from the urothelium into the lamina propria, but has not invaded into the muscle layer of the bladder. Unfortunately, recurrence frequently occurs in NMIBC (50-70%) (27, 28), requiring lifelong surveillance and making it the most expensive cancer to treat on a per patient basis (29). In addition, some of these tumors (10-20%) may progress into MIBC (28, 30). While non-muscle invasive tumors have a relatively high 5-year survival rate (88-98%), tumors that end up invading through the muscle layer (T2) significantly decreases the survival rate to about 63%. This decreases further to 46% in stage 3, where the tumor has invaded into the underlining fat layer surrounding the bladder, and 15% for stage 4, where the cancer has spread to nearby reproductive organs or to distant parts of the body. Common sites of metastases include lung, liver, and bone and are often hard to treat (31).

**Molecular Pathology**

Due to the recurrent nature of these tumors and the ability of some NMIBC to progress and invade into the muscle, constant surveillance is crucial in bladder cancer. Many efforts have been made towards defining the mechanisms behind both NMIBC and MIBC in attempts to identify biomarkers or new therapeutic targets that can help manage and treat the disease. Studies have found particular genetic alterations/characteristics that have a greater propensity to be shown in one type than the other. Non-muscle invasive tumors tend to be associated with fibroblast growth factor receptor 3 (FGFR3) (32, 33, 34) whereas muscle invasive tumors often show loss of commonly mutated tumor suppressor gene p53 and retinoblastoma (RB) function (35, 36, 37), which suggests that NMIBC and MIBC may arise and progress along distinct pathways. Fibroblast growth factor receptor 3 (FGFR3) is a receptor tyrosine kinase that is

involved in development, cell growth, differentiation, proliferation, and angiogenesis. Receptor binding by its ligand results in the autophosphorylation of the receptor that activates downstream RAS/MAPK and phosphatidylinositol 3-kinases (PI3K) signaling. In a study of urothelial cell carcinomas, 59% displayed FGFR3 mutations that was related to low stage/grade tumors (38).

In the case of MIBC, p53 and RB genes are frequently altered in urothelial carcinoma, which is associated with a significant decrease in metastasis-free survival of patients (39). The p53 tumor suppressor protein can inhibit cell cycle when DNA damage has been detected and even trigger apoptosis when the damage is unrepairable (40). Alterations in this gene have been reported to be involved with bladder cancer progression with over 50% of the bladder tumors containing a p53 mutation (35). It has also been reported that FGFR3 and p53 alterations are mutually exclusive with p53 mutation detected in the high stage and high grade tumors (38, 41). Similarly, the RB gene also regulates cell growth by inhibiting the cell cycle process through its ability to control the G1 checkpoint and block entry into S-phase (42). RB and p53 alterations are thought to work together, rather than individually, to promote MIBC (35, 43).

Overexpression of the epidermal growth factor receptor (EGFR) is another commonly observed alteration in MIBC and is associated with reduced recurrence-free and overall survival (44). EGFR is also a receptor tyrosine kinase and activation of the receptor results in downstream RAS/MAPK and PI3K signaling; thus, leading to continued cell growth/proliferation and survival of the cancer cells. Additionally, it has been reported that MIBC arise from Sonic hedgehog (Shh)-expressing stem cells (45). This study displayed the initiation and progression of bladder cancer upon exposure to N-butyl-N-4-hydroxybutyl nitrosamine (BBN) carcinogen in a mouse model. Invasive bladder carcinoma was induced by BBN and was found to arise from basal stem cells that express *Shh*. BBN exposure had caused the accumulation of mutations that

would lead to CIS formation after clonal expansion of these *Shh*+ basal cells. However, upon the establishment of muscle invasive carcinoma from the once CIS lesion, *Shh* expression was found to be lost. This loss of *Shh* attributes to the reduction of bladder urothelium differentiation-promoting factors bone morphogenic protein 4 and 5 (BMP4, BMP5) expression in the stroma, which in turns can potentiate tumor progression (46).

Recently, large scale studies have been conducted investigating the gene expression of different bladder tumors in attempts to identify molecular signatures that can help predict disease recurrence, progression, and the course of action. These studies have revealed additional insight into the molecularly distinct nature of UC and broadened our understanding of bladder cancer beyond the classic NMIBC vs. MIBC group distinction. Lindgren D et al. (47) conducted a gene expression analysis of UC with the aim to refine the classification system of bladder tumors. Their analysis resulted in the identification of 2 molecular subtypes: MS1 and MS2. MS1 were characterized by high FGFR3 and PIK3CA mutations and encompassed the Ta tumors, while MS2 displayed genomic instability that could be attributed to loss of RB1, E2F3 amplification, and p53/MDM2 alterations. This subtype encompassed the MIBC that showed aggressive growth and poor prognosis. As for the T1s, they were equally distributed amongst MS1 and MS2. Later on, these two MS groups were further divided into additional subtypes that could better predict the nature of the disease and its propensity to progress and invade. Based on gene expression profiling, five subgroups were identified similar to the molecular classification system of breast cancer: Urobasal A, genomically unstable, urobasal B, squamous cell carcinoma-like, and a heterogenous infiltrated class of tumors (48). These subtypes not only presented distinct molecular characteristics, but also displayed distinct clinical outcomes. Urobasal A mostly resembled the same patterns of normal urothelium in regards to their expression of keratins-5, -

13,-15, and -17 and these tumors were of low grade NMIBC. Both urobasal A and urobasal B grouped tumors displayed high FGFR3 mutations and elevated CCND1 and p63 expression; however, while urobasal A was associated with a good prognosis, urobasal B showed the opposite with the worst prognosis. This could be explained by the frequent p53 mutations observed in the urobasal B tumors, potentially accounting for 50% of the muscle invasive tumors present in this group. Therefore, it was proposed that the urobasal B subtype may have progressed from urobasal A. The genomically unstable subtype also contained high p53 mutations as well as cyclin E and ERBB2 expression. They had low cytokeratin expression and represented a high-risk group (~40% MIBC) that displayed intermediate prognosis. Squamous cell carcinoma-like subtype was the most strikingly different from the others, characterized by high expression of basal keratins that are not normally expressed in the urothelium (KRT4, KRT6, KRT14, and KRT16) and connected to poor prognosis. Lastly, the infiltrated tumor subgroup was distinguished by upregulation of immune response signature and represented a heterogenous class of tumors that contained features of genomically unstable, urobasal B, and squamous cell carcinoma-like, thus, showing an intermediate prognosis.

Furthermore, subsequent gene expression profiling studies had categorized MIBC into distinct intrinsic molecular subtypes: basal and luminal based on their resemblance to basal and luminal breast cancer subtypes (49, 50). Damrauer JS et al. had created a meta-dataset of 262 high-grade muscle invasive tumors that was clustered into two groups. Whereas the basal-like subtype had high expression of high molecular weight keratins (KRT14, KRT5, KRT6B) and alterations in the RB pathway, the luminal subtype was characterized by high uroplakin (URP1B, URP2, URP3A), keratin 20, and enriched with FGFR3 and tuberous sclerosis-1 (TSC1) mutations. In accordance with these molecular features, basal-like subtype had a poor overall

survival compared to the luminal. A p53-like luminal classification was added in by Choi W et al. as these tumors expressed luminal markers, but also contained a wild-type p53 gene expression signature (50). A special feature of this group is that they tend to display chemoresistance. In this study, the luminal subtype was found to contain active peroxisome proliferator-activated receptor gamma (PPARγ) and was enriched with FGFR3 mutations as well as the luminal markers: CD24, FOXA1, GATA3, ERBB2, ERBB3, XBP1 and KRT20. The basal subtype expressed CD44, KRT5, KRT6, KRT14, CDH3, high levels of EGFR, p63 activation, and showed a much more aggressive phenotype. These basal tumors were also characterized by squamous differentiation, which corresponded to the squamous cell carcinoma subtype that was identified by Sjödahl G et al. (48). In addition, The Cancer Genome Atlas Research (TCGA) conducted an integrated study of 131 high-grade muscle invasive bladder carcinomas that also revealed four group clusters of which closely resembled that of the basal, luminal, and p53-like subtypes (51). With significant overlap between these different studies, it seems to be pretty clear of the existence of different urothelial carcinoma subtypes, much like in breast cancer, that could potentially help establish a method of efficiently identifying and treating these particular subgroups.

Analogously, a large study with RNA sequencing of 460 NMIBC also revealed the existence of both basal and luminal subtypes in these tumors similar to what had been seen in MIBC (52). Non-muscle invasive tumors were separated into three different classes: Class 1 mainly consistent of papillary tumors and thus had the best prognosis. They were characterized by early cell-cycle gene expression and activating mutations of FGFR3. On the other hand, class 2 included CIS and had high expression of late cell-cycle genes. Class 3 displayed high expression of cytokeratins (KRT5/14) as well as CD44, a bladder cancer stem cell marker.

Tumors in classes 1 and 2 were found to be similar to luminal and class 3 to basal, displaying significant overlap with the previously mentioned studies. The high grade T1s were seen more frequently in classes 2 and 3, but majority of the disease progression was observed in class 2, suggesting that the class 2 tumors could signify the ones that are at an increased risk for invading into the muscle. This classification system could be useful in predicting the course of action for these recurrent non-muscle invasive bladder tumors.

Overall, these clustering and gene expression studies led to the identification of distinct bladder cancer subtypes that help in gaining a better understanding of the disease. However, it remains to be determined how these subtypes will actually be translated into predictive factors to monitor and treat bladder cancers. Aside from FGFR3 and p53 mutations being consistently reported, there is now a wider picture of urothelial carcinoma including many additional genes that can be incorporated with what is currently known. On the other hand, further studies are needed to dissect out the molecular mechanisms behind these different subtypes and how these particular gene signatures actually play into tumor recurrence, progression, and chemoresistance to be able to discover the best way to utilize and therapeutically target these genes.

**Diagnosis/Treatment**

The earliest clinicopathologic sign of bladder cancer may be the presence of hematuria, although this can be caused by many other factors (i.e. infections, kidney stones). Cystoscopy examination is traditionally used to diagnose bladder cancer, including histological evaluation of the tumor biopsy. In some cases, urine cytology can be used to examine for the presence of cancer cells or other imaging testing, such as intravenous pyelogram or CT scan, can be performed to visualize the urinary tract and its surrounding tissues. Bladder cancer is typically treated based on tumor stage, although the size and grade can be factored in as well. In most

cases, NMIBC is treated with transurethral resection (TURBT) and can be followed by intravesical therapy, which can include Bacille-Calmette Guerin (BCG), mitomycin, or chemotherapeutic drugs. In fact, intravesical therapy with BCG, mitomycin C, doxorubicin, and epirubicin were found to decrease the risk of recurrence compared to no intravesical therapy (53). Particularly with CIS and intermediate to high risk papillary tumors, intravesical BCG significantly reduces the risk of progression after TURBT (54). Nevertheless, due to the high incidence of recurrence, follow-up testing is recommended every 3 to 6 months after treatment.

For MIBC, TURBT is typically used to help determine the extent of the tumor rather than a means to treat it, instead partial or radical cystectomy is recommended. The evaluation of radical cystectomy as the treatment of MIBC from a large group of patients had revealed that long-term survival and low local recurrence can be achieved (55). Neoadjuvant or adjuvant chemotherapy is also given to lower the chance of the cancer returning, with pre-surgical cisplatin-based chemotherapy being the standard of care for high risk MIBC (56). The use of neoadjuvant chemotherapy has been shown to improve survival (77 months vs. 46 months for cystectomy alone) and increase the likelihood of eliminating residual cancer in the cystectomy specimen (57). Meta-analysis of patients who had undergone platinum-based combination neoadjuvant chemotherapy with local treatment vs. local treatment alone showed a significant improvement in survival and disease-free survival (58). In a randomized trial of radical cystectomy versus cisplatin, vinblastine, and methotrexate chemotherapy treatment after radical cystectomy, the adjuvant group consistently showed longer survival (median 63 months for adjuvant group vs. 36 months for no chemo) and high progression-free disease (37 months for adjuvant vs. 12 months for none) (59). A combination of drugs consisting of methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) has been the standard treatment for bladder

cancer (60). However, there are reports of gemcitabine plus cisplatin (GC) treatment working just as well as MVAC treatment with less adverse side effects and comparable survival rates (61, 62, 63). Whereas radical cystectomy is the standard treatment for tumors that are stage 3, the goal of stage 4 tumors is to shrink or prevent the growth of the tumor using chemotherapy. If chemotherapy does not appear to be a good option for the patient, radiation therapy is usually given as an alternative. For those recurrent tumors, combination chemotherapy regiments, GC or MVAC are given as treatment.

Routine follow-up testing after treatment typically involves cystoscopy and urine cytology, which still has the risk of missing papillary tumors and high-grade flat CIS lesions (64). No standard method of non-invasive testing other than urine cytology is currently recommended and the frequent monitoring makes it difficult for patients even after successful treatment. Even though many efforts have been made to understand the genetics and molecular mechanisms behind UC, a biomarker has yet to be identified to help detect different subtypes of bladder cancer or possible recurrence/disease progression. Urine-based tests have been proposed. Five of them (NMP22®, BTA®, UroVysion® FISH, ImmunoCyt™, and Cxbladder™) have been approved by the FDA and /or commercially available, but have not been proven to work better than cystoscopy examination (65).

Treatment selection is largely dependent on the clinical pathology/stage of the tumor, which can be hard to clearly determine, especially in regards to the recurrent nature of the tumor. This really demonstrates the need for further investigation underlining the mechanisms in bladder cancer progression and invasion for the development of improved and more feasible detection and treatment methods. There have been minimal therapeutic advances in treating MIBC in the past 20 years until recently when the FDA approved of a new immunotherapy drug

atezolizumab (Tecentriq®) based on a phase II trial with 310 patients (66). The results of the trial showed anti-tumor response (with 17% showing tumor shrinkage) in patients whose disease had progressed after failing to respond to platinum-based chemotherapy. It was tolerated seemingly well by the patients with fatigue being the most common side effect. Atezolizumab, a humanized immunoglobulin G1 monoclonal antibody, targets the programmed death-ligand 1 (PD-L1) protein and prevents binding to its receptor programmed cell death protein 1 (PD-1), present on T cells. PD-L1 binding to PD-1 suppresses the immune response and thus considered immune "checkpoint" proteins. The study also reported that although the responses were found across all the TCGA subtypes, it was significantly higher in the luminal cluster II subtype, showing that the utilization of the identified subtypes can be of informative use when predicting treatment response in patients. Therefore, with this newly approved therapy and the revelation of the existence of different subtypes of bladder cancer, the development of better screening and treatment methods will probably be emerging.

**Summary-**

Urothelial carcinoma is the most common bladder cancer, affecting 3 times as many men than women. With smoking accounting for 50% of the bladder cancer cases, it is a disease that reveals the importance of both environmental and genetic factors in the development of bladder cancer. Bladder cancer is typically categorized as NMIBC vs. MIBC depending on the depth of growth of the tumor in the urothelium. Approximately 75-85% are NMIBC at initial diagnosis, which has a good 5-year survival rate (88-98%). However, the high rate of recurrence (50-70%) requires constant long-term surveillance that leaves a tremendous burden on both the patient and health care system. In addition, 10-20% of the NMIBC has the risk for progressing into MIBC, which significantly reduces the survival rate. Many efforts have been made in gaining a better understanding of the progression and recurrence of bladder cancer, particularly large scale gene expression studies have resulted in the identification of basal and luminal subtypes that can help predict the nature of the disease based on different genetic signatures of the tumor. Further studies are needed to truly understand how the molecular characteristics of these subtypes contribute to bladder cancer and how they will translate into the clinic. However, these recent studies are moving towards the development of better screening and detection methods as well as therapeutic targets and predictors of response for bladder cancer.

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## Chapter II

## Identifying genes that regulate bladder cancer invasion

**Abstract-**

Defining differences that underlie the mechanisms that promote a muscle invasive phenotype in bladder cancer is challenging. In this study, we addressed this challenge by determining if there are specific gene expression differences between patient-derived T1 vs. T2 bladder cancers. RNA sequencing of T1 and T2 patient tissue samples revealed genes that were differentially expressed between the two groups. Additionally, the Oncomine database was examined to further narrow down potential candidates that differentiate T1s from T2s. This led to the identification of four potential candidate genes that may be involved in muscle invasive bladder cancer: *Fibulin-3* (*EFEMP1*), *DUSP2*, *glut-3* (*SLC2A3*), and *GPR126*. Validation of these genes by both qRT-PCR and western blotting amongst the T24, UMUC-13, UMUC-3, RT4, and 5637 bladder cancer cell lines revealed fibulin-3, which was highly expressed in the T2 tissues than the T1s, to be a likely candidate in regulating bladder cancer invasion. Whereas the most invasive cell lines, T24 and UMUC-13, demonstrated the highest fibulin-3 expression, the least invasive cells, RT4 and 5637, demonstrated the lowest fibulin-3 expression, suggesting a correlation between fibulin-3 expression and bladder cancer invasion. To further validate these findings, fibulin-3 expression was measured using qRT-PCR from formalin fixed paraffin embedded tissues from patient bladder tumor samples ranging from stages Ta-T4. These studies

confirmed elevated fibulin-3 expression in muscle invasive compared to non-muscle invasive bladder cancer validating the approach taken to identify differentially expressed genes in urothelial cancer of varying invasive potential.

**Introduction-**

Bladder cancer is the eighth most common cause of cancer death in men. For 2016, it is estimated that 76,960 new cases of bladder cancer will be diagnosed and 16,390 deaths will arise due to the disease (1). Urothelial carcinoma (UC) is the most common type of bladder cancer and can be categorized as either non-muscle invasive bladder cancer (NMIBC), stages Ta-T1 or muscle invasive bladder cancer (MIBC), stages ≥T2. In majority of the cases, patients are first diagnosed with stages Ta-T1 (2). However, the recurrence rates of these tumors are high, requiring lifelong surveillance and making it one of the most expensive cancers to treat on a per patient basis (3). In addition to non-muscle invasive recurrence, Ta-T1 tumors may progress into MIBC. While non-muscle invasive tumors have a relatively high 5-year survival rate (88-98%), tumors that progress to muscle invasion result in a decreased survival rate (approximately 63% at 5-years). Post-treatment monitoring typically involves cystoscopy and urine cytology, which still has the risk of missing papillary tumors and high-grade flat carcinoma in situ CIS lesions (4). Currently, there are no highly effective standard methods of non-invasive testing other than the urine cytology that is recommended (5) and the frequent monitoring is challenging for patients even after successful treatment. Thus, investigating the mechanisms underlying bladder cancer progression to a muscle invasive phenotype is imperative to develop improved and facile detection and treatment methods. Our objective of this study was to determine if there are genes that are differentially expressed between T1 vs. T2 bladder cancer as we hypothesize that specific gene signatures between T1 and T2 bladder cancer can help identify key regulators in bladder cancer progression and invasion.

**Materials and Methods-**

*RNA Sequencing*

RNA was isolated from seven T1 and seven T2 bladder cancer patient samples. Patients were all from the University of Michigan, Ann Arbor. Bladder cancer tissues were obtained from cystectomy samples, which were placed in OCT and snap frozen with liquid nitrogen. Paired end RNA sequencing libraries were prepared and sequenced using an Illumina HiSeq by the University of Michigan DNA Sequencing Core. Analysis of the sequencing data was performed with Tophat and Cufflinks to obtain gene expression values (units FPKM). Further analysis with the gene expression values was done with Significance Analysis of Microarrays (SAM) to detect differentially expressed genes comparing all the T1s with the T2s.

*Cell Lines*

T24, UMUC-13, UMUC-3, 5637, and RT4 cell lines were generously provided by Dr. Monica Liebert (University of Michigan, Ann Arbor). All cells, except RT4, were maintained in Dulbecco's Modified Eagle Medium, DMEM, high glucose (Gibco-Life Technologies (GIBCO), Carlsbad, CA) supplemented with 10% fetal bovine serum, 100U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% $CO_2$ at $37^0$C. RT4 cells were maintained in DMEM-F12 (GIBCO) complete medium. Cell lines were authenticated using short tandem repeats and were evaluated monthly to ensure they were Mycoplasma free.

*RNA Extraction/qRT-PCR*

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) and purified with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA concentrations were measured using a Nanodrop Spectrophotometer ND-1000 (Wilmington, DE). 1 μg of total RNA was used for reverse transcription using SuperScript III First-Strand Synthesis System (Invitrogen). qRT-PCR was performed in triplicates using SYBR

Green (Qiagen) with the LightCycler 480 instrument (Roche, Indianapolis, IN). Primers were chosen using Primer-BLAST and ordered from Invitrogen (Table 2.1).

For qRT-PCR performed to confirm fibulin-3 expression in the non-muscle invasive vs. muscle invasive patient bladder tumors, RNA from FFPE tissues was extracted using the AllPrep DNA/RNA FFPE kit (Qiagen). Gene –specific priming with the reverse primers of fibulin-3 and β-actin was used to create cDNA (500ng RNA). Primer/probe sequences were designed using PrimerQuest and ordered from IDT (Coralville, IA) (Table 2.1). Reactions were run in triplicates using TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA). Fibulin-3 expression was normalized to β-actin expression for each sample.

*Western Blotting*

Cells were lysed with RIPA buffer complemented with protease inhibitor cocktail, PMSF, NaF, and $Na_3VO_4$, collected into a 1.5ml Eppendorf tube, and placed on ice and vortexed intermittently for 20 minutes. After centrifugation at 15,000rpm for 15 minutes at $4^0$C, supernatant was collected and measured for protein concentration using Pierce™ BCA Protein Assay (Thermo Fisher Scientific, Carlsbad, CA). 30-50μg of protein was resolved in a 4-20% gradient Novex™ Tris-Glycine gel (Thermo Fisher Scientific) under reducing conditions and transferred onto an Immun-Blot PVDF membrane (BioRad, Hercules, CA). Appropriate primary antibody was added after blocking for an hour with 5% non-fat milk: anti-fibulin-3 (TA503772, Origene, Rockville, MD), anti-DUSP2 (sc-32776, Santa Cruz, Santa Cruz, CA), anti-glut3 (GT33-A, Alpha Diagnostic Intl., San Antonio, TX), anti-GPR126 (ab75356, abcam, Cambridge, UK), and anti-β-actin (A5441, Sigma, St. Louis, MO). The antigen-antibody reaction was detected using the appropriate horseradish peroxidase-conjugated secondary antibody, followed by visualization with the electrochemiluminescence detection system using film.

*Statistical Analysis*

Experiments were repeated two to three times and statistically analyzed using GraphPad Prism 6. Pearson correlation was used to determine correlation between two variables and the comparison of two groups was analyzed using unpaired t-test. p-values that were less than 0.05 were considered significant.

| Gene | Primer Sequence (5'-3') |
|---|---|
| Fibulin-3 | F: TGA GCT AAG CAG TGA CAG GC<br>R: GTA TCC CTG GGG GCA CAT AC |
| DUSP2 | F: TAC TTC CTG CGA GGA GGG TT<br>R: GGC TGG TTT TGT CCC CTG TT |
| Glut-3 | F: GAG GTG CTG CTC ACG TCT CT<br>R: TGA ATT GCG CCT GCC AAA GC |
| GPR126 | F: GAG GGG GAC CCA AGT ACC TA<br>R: GAG GAA GTA GGG TGT GCG TG |
| β-actin | F: GAT GAG ATT GGC ATG GCT TT<br>R: CAC CTT CAC CGT TCC AGT TT |

| Gene | IDT Primer/Probe (5'-3') |
|---|---|
| Fibulin-3 | F: CAC CAG GCT CAT TTT ATT GCC<br>R: GAT TGC TGG CAT CAC ATT CAT<br>P: /56-FAM/TGC AGT CCT /ZEN/GGG TTT CAA TTG GC/3IABkFQ/ |
| β-actin | F: GGA TGC AGA AGG AGA TCA CTG<br>R: CGA TCC ACA CGG AGT ACT TG<br>P: /56-FAM/CTT GAT CTT /ZEN/CAT TGT GCT GGG TGC C/3IABkFQ/ |

**Table 2.1-List of primers used in qRT-PCR experiments.** Primers were designed either using Primer-Blast (top) for cell lines or PrimerQuest (bottom) for the patient tissues.

**Results-**

*Differential expressed genes between T1 vs. T2 bladder cancer*

To determine if there are specific genes that are key regulators of muscle invasion, we sought to identify genes that are differentially expression between T1 vs. T2 bladder tumors (analysis schema in Fig. 2.1). To achieve this, we performed RNA-Seq of seven T1 and seven T2 patient bladder cancer tissues. After obtaining the expression values using Tophat and Cufflinks, we conducted significance analysis of microarrays (SAM) to nominate differentially expressed genes between all T1 against all T2 bladder cancers. From this analysis, we prioritized a list of 32 genes that were not only differentially expressed between the two groups, but could potential play a role in regulating MIBC based on the significance and the fold increase as well as potential novelty of the gene in bladder cancer (Table 2.2). These genes were then searched for and examined in the Oncomine database across different bladder cancer studies, which helped us narrow down and identify potential genes that may be involved in bladder tumor progression or could serve as markers of MIBC.

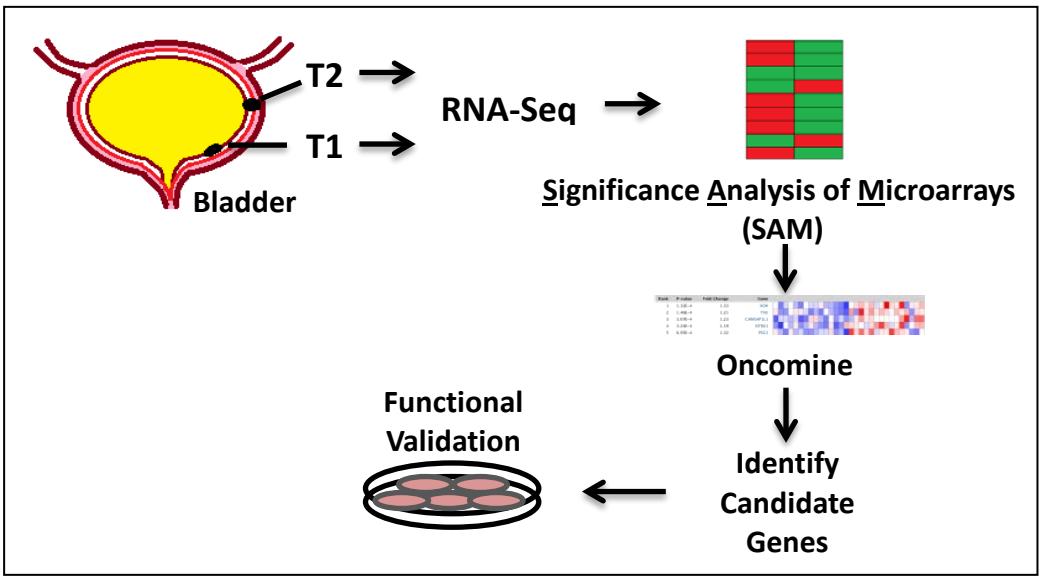


**Figure 2.1-Identification of candidate genes.**
Schematic approach taken to identify potential genes involved in muscle invasive bladder cancer.

| Gene ID | Gene Name | Score(d) | Numerator(r) | Denominator(s+s0) | Fold Change | q-value(%) |
|---|---|---|---|---|---|---|
| SFRP2 | ENSG00000145423 | 2.1 | 51.3 | 24.0 | 44.1 | 16.3 |
| MFAP5 | ENSG00000197614 | 2.9 | 17.5 | 6.0 | 14.5 | 10.3 |
| KLK6 | ENSG00000167755 | 1.1 | 15.2 | 13.8 | 13.3 | 37.9 |
| KLK5 | ENSG00000167754 | 1.0 | 13.0 | 12.5 | 12.7 | 43.7 |
| SLC2A3 | ENSG00000059804 | 1.7 | 100.7 | 59.9 | 12.6 | 24.7 |
| MMP12 | ENSG00000110347 | 2.2 | 34.2 | 15.9 | 12.5 | 16.3 |
| MMP11 | ENSG00000099953 | 2.0 | 105.1 | 52.4 | 11.2 | 17.7 |
| ADAMDEC1 | ENSG00000134028 | 2.4 | 19.9 | 8.5 | 9.7 | 14.0 |
| FGF7 | ENSG00000140285 | 1.9 | 10.1 | 5.3 | 9.1 | 20.3 |
| FADS2 | ENSG00000134824 | 2.8 | 67.4 | 24.0 | 8.0 | 11.1 |
| EFEMP1 | ENSG00000115380 | 3.6 | 28.8 | 8.0 | 6.1 | 9.0 |
| PRRX1 | ENSG00000116132 | 3.7 | 7.8 | 2.1 | 5.1 | 9.0 |
| LEPREL2 | ENSG00000110811 | 4.9 | 14.0 | 2.9 | 5.0 | 4.7 |
| GPR34 | ENSG00000171659 | 3.3 | 5.3 | 1.6 | 3.6 | 9.9 |
| PDLIM2 | ENSG00000120913 | 3.3 | 23.5 | 7.1 | 3.5 | 9.9 |
| MARVELD1 | ENSG00000155254 | 7.0 | 14.0 | 2.0 | 3.3 | 0.0 |
| HOXB8 | ENSG00000120068 | -2.2 | -22.3 | 10.2 | 0.1 | 14.0 |
| DUSP2 | ENSG00000158050 | -2.2 | -65.4 | 29.1 | 0.1 | 13.3 |
| DMBX1 | ENSG00000197587 | -1.3 | -12.4 | 9.9 | 0.1 | 36.4 |
| HOXB6 | ENSG00000108511 | -2.5 | -41.0 | 16.1 | 0.1 | 10.3 |
| HOXB5 | ENSG00000120075 | -2.4 | -25.5 | 10.8 | 0.2 | 11.8 |
| PAX8 | ENSG00000125618 | -1.0 | -23.2 | 23.5 | 0.2 | 50.1 |
| ERN2 | ENSG00000134398 | -1.5 | -38.2 | 26.3 | 0.2 | 30.6 |
| SCUBE2 | ENSG00000175356 | -2.0 | -64.1 | 32.7 | 0.2 | 19.2 |
| SHROOM1 | ENSG00000164403 | -3.0 | -61.7 | 20.3 | 0.2 | 6.0 |
| AZGP1 | ENSG00000160862 | -2.2 | -11.2 | 5.1 | 0.2 | 14.0 |
| C11orf92 | ENSG00000196167 | -2.9 | -9.9 | 3.4 | 0.2 | 7.2 |
| SPINK1 | ENSG00000164266 | -2.5 | -1963.2 | 798.4 | 0.2 | 11.1 |
| TBX6 | ENSG00000149922 | -2.2 | -15.1 | 7.0 | 0.2 | 15.1 |
| GATA3 | ENSG00000107485 | -2.9 | -125.6 | 42.8 | 0.2 | 7.2 |
| GPR126 | ENSG00000112414 | -2.3 | -16.9 | 7.5 | 0.2 | 13.3 |
| FOXQ1 | ENSG00000164379 | -3.6 | -83.5 | 23.5 | 0.2 | 4.7 |

**Table 2.2-Genes differentially expressed between T1 vs. T2.**

List of genes that were identified to be differentially expressed between the T1 vs. T2 bladder tissue samples based on the significance, fold increase, and novelty of these genes in bladder cancer. Genes that had consistent data across bladder cancer studies in Oncomine and prioritized for validation are highlighted in yellow. Values were obtained from SAM analysis with RNA-Seq results.

*Identification of four potential gene candidates*

These efforts led to the prioritization of four novel candidate genes drivers of invasive urothelial carcinoma and/or potential biomarkers of highly invasive carcinoma: *Fibulin-3* (*EFEMP1*), *DUSP2*, *glut3* (*SLC2A3*), and *GPR126*. *Fibulin-3* and *glut3* expressions were found to be higher in the T2 patients than the T1s and in more aggressive vs. less aggressive urothelial cancer in Oncomine, whereas *DUSP2* and *GPR126* were lower in the T2s than the T1s and lower in more aggressive vs. less aggressive urothelial carcinoma in Oncomine (Fig. 2.2A). To validate these findings, fibulin-3, DUSP2, glut3, and GPR126 were evaluated for transcript and protein expression in various bladder cancer cell lines that ranged from transitional papilloma to high grade transitional cell carcinoma. qRT-PCR and western blotting revealed fibulin-3 to be highly expressed in the high grade TCC-T24 and lymphatic metastases of TCC-UMUC-13 and contained lower expression in the lower grade-5637 and transitional papilloma-RT4 cell lines (Fig. 2.2B), confirming the integrative expression analysis and supporting our follow-up study. Interestingly, DUSP2 showed the opposite expression pattern, showing higher protein expression in RT4 and 5637. Glut3 and GPR126 expression did not show concordant expression with invasive status of the cell lines and thus were not considered further for validation.

**A**

| | | T1 | | | | | | | T2 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ensembl_id | gene | #6059 | #774 | #727 | #4849 | #1335 | #6135 | #6910 | #975 | #879 | #1530 | #3948 | #4945 | #4474 | #1041 |
| ENSG00000115380 | Fibulin-3 | 0.5 | 4.3 | 0.7 | 21.1 | 0.8 | 4.1 | 0.7 | 35.8 | 56.8 | 34.1 | 15.5 | 38.8 | 52 | 0.7 |
| ENSG00000158050 | DUSP2 | 127.5 | 72.9 | 54.4 | 1.7 | 39 | 219.8 | 8.5 | 8.7 | 7 | 25.6 | 2.9 | 11.2 | 7.2 | 3.5 |
| ENSG00000059804 | Glut3 | 3.6 | 5.7 | 3.1 | 1.9 | 10.8 | 23.4 | 5.4 | 416 | 74.9 | 8.9 | 231.4 | 10.5 | 11.7 | 5.3 |
| ENSG00000112414 | GPR126 | 7.7 | 23.2 | 3.3 | 58.5 | 30.2 | 13.6 | 12.9 | 0.8 | 1.1 | 18.1 | 2.1 | 1.5 | 4.9 | 2.4 |

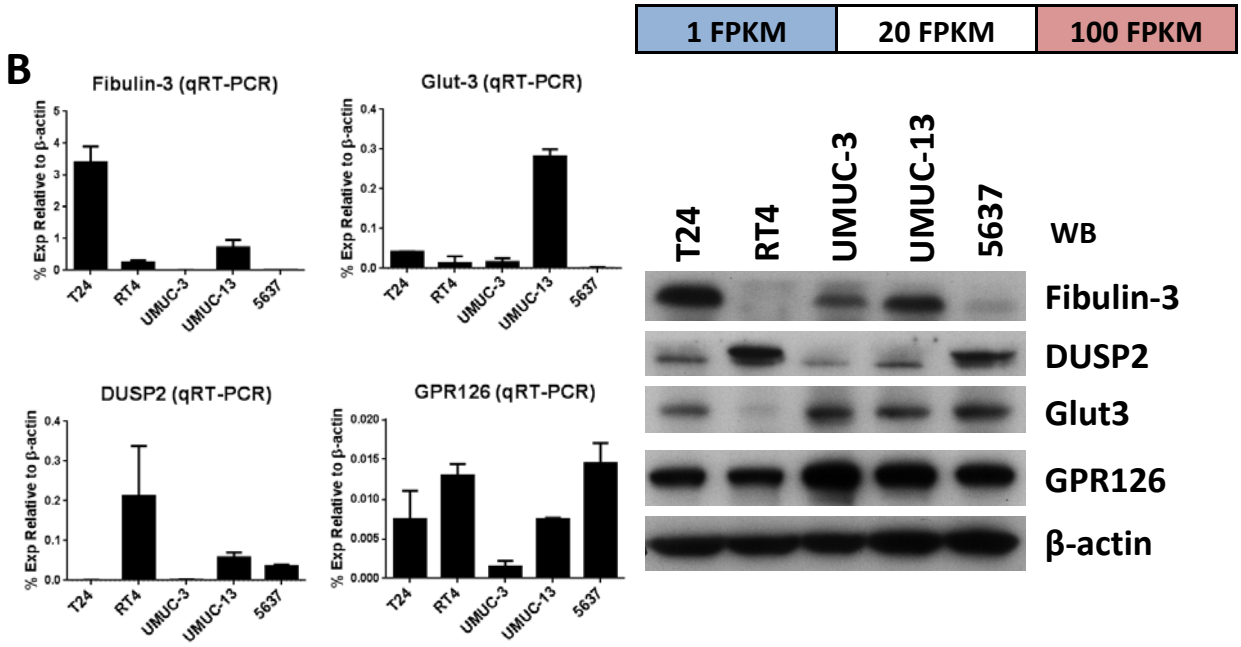


**Figure 2.2.-Validation of the candidate genes with bladder cancer cell lines.**

**A**) T1 and T2 bladder cancer tissues (n=7 each) were subjected to RNA-Seq. Expression values (in Fragments Per Kilobase of transcript per Million mapped reads [FPKM]) of prioritized differentially expressed genes. **B**) To measure mRNA, total RNA was extracted from different bladder cancer cell lines (T24, RT4, UMUC-3, UMUC-13, 5637) and 1 μg of total RNA was reverse transcribed to make cDNA. Human primer pairs of the candidate genes were designed and subjected to real time qRT-PCR along with β-actin (loading control). Results are reported as means from three separate experiments. To measure protein, 30 μg of total protein were loaded in a 4-20% tris-glycine gel and subjected to SDS-PAGE, transferred to a PVDF membrane, and probed with anti-human fibulin-3, DUSP2, glut-3, and GPR126 antibody. β-actin was used as loading control.

*Fibulin-3 expression correlates with bladder cancer invasion*

Therefore, we focused on studying fibulin-3 and understanding the role it may play in bladder cancer invasion and/or progression. To determine if the increase fibulin-3 expression correlated with the invasive phenotype, we first evaluated the invasive and migratory potentials of the bladder cancer cell lines. T24 and UMUC-13 cells had the greatest invasive and migratory potential; whereas, the 5637 and RT4 cells had the least (Fig. 2.3A). We then correlated the invasiveness of these cells with their fibulin-3 expression and found a correlation between fibulin-3 expression and invasion (Fig. 2.3B).

*Fibulin-3 expression is higher in MIBC than NMIBC*

For further validation of the RNA-Seq findings and to demonstrate their potential clinical relevance, fibulin-3 expression was evaluated using qRT-PCR of patient bladder cancer samples ranging from stages Ta-T4. Specimens were macrodissected to enrich for high tumor content as needed. Fibulin-3 expression was elevated in MIBC compared to NMIBC thus validating the RNA-Seq results (Fig. 2.4).

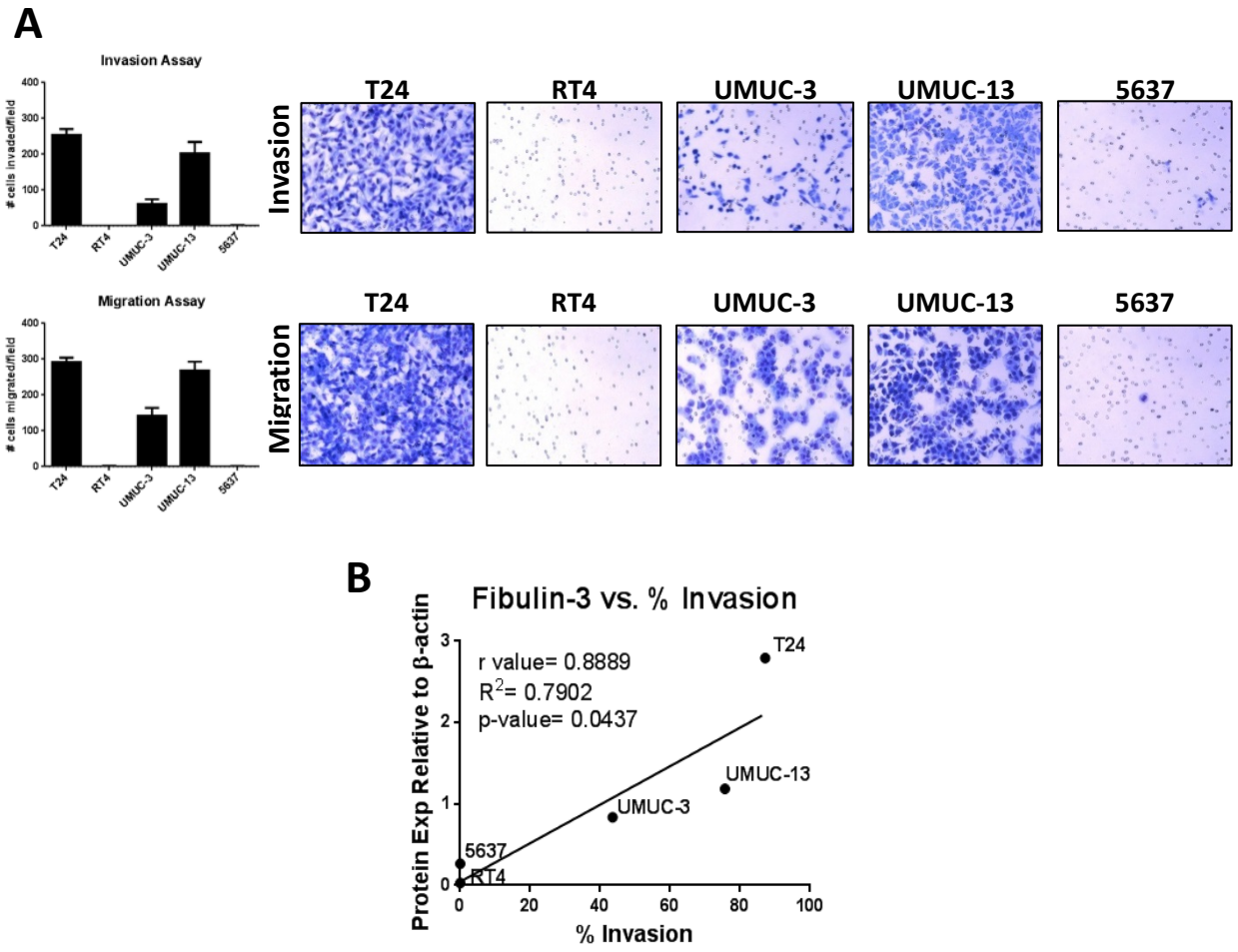


**Figure 2.3-Fibulin-3 expression correlates with invasion.**

**A**) Different bladder cancer cell lines (T24, RT4, UMUC-3, UMUC-13, and 5637) were assayed for their invasive and migratory potentials using transwell inserts. 1 x $10^5$ cells/transwell were plated in the upper chamber in serum-free media conditions with 10% FBS conditions serving as the chemoattractant. After 24 hours, cells that invaded/migrated were fixed and stained. Cells were counted in five random 20X microscopic fields. Results are shown as the mean number of cells that migrated/invaded through the transwell membrane. Representative images of the fields (20X) that were counted are shown. **B**) Densitometry measurements of Western blot bands (from Fig. 2.3B) were made using ImageJ to quantify relative protein expression of fibulin-3 relative to β-actin. Fibulin-3 protein expression of each bladder cancer cell line was plotted against that cell's invasiveness (%) (% invasion = number of cells invaded/number of cells migrated X 100); Pearson correlation coefficient.

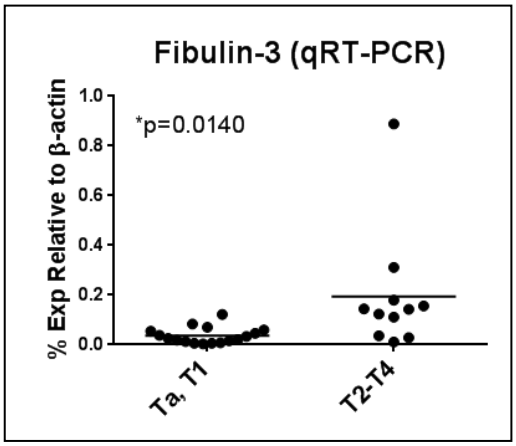


**Figure 2.4-Fibulin-3 is overexpressed in MIBC vs. NMIBC.**
RNA was extracted from both non-muscle (n=17) and muscle invasive bladder cancer (n=11) formalin-fixed paraffin-embedded patient tissues and subjected to qRT-PCR for *fibulin-3* transcriptional expression normalized to β-actin; unpaired two-sided t-test.

**Discussion-**

Many efforts have been made recently in understanding the underlying mechanisms behind both NMIBC and MIBC. These include reports of frequent mutations in UC (6, 7, 8) to identification of distinct bladder cancer subtypes that indicate their propensities to be aggressive and invade to surrounding tissues or even predict drug resistance (9, 10, 11). However, the key drivers and steps involved in progression from NMIBC to MIBC are still poorly understood. The goal of the current study was to identify genes that were differentially expressed between T1 and T2 urothelial cancer that could be further studied to identify a molecular mechanism that contributes to the conversion of non-muscle invasive to MIBC. We approached this by evaluating differentially expressed genes between T1 and T2 patient tissue samples. Four candidate genes were chosen that not only were differentially expressed between T1s vs. T2s, but also were consistently up- or down- regulated across urothelial carcinoma studies in the

Oncomine database: *Fibulin-3* and *glut-3* were overexpressed, whereas *DUSP2* and *GPR126* were downregulated in MIBC. Validation of these results with various bladder cancer cell lines led us to discover higher fibulin-3 expression in the higher grade TCC cell lines that directly correlated with their invasive and migratory potential. Thus, we were inclined to continue pursuing fibulin-3 in our study as results indicated it to be a potential gene involved in bladder cancer invasion. This was further verified in our Ta-T4 formalin fixed paraffin embedded patient samples where we had also observed higher *fibulin-3* expression in MIBC compared to the NMIBC. Additional studies would be needed to confirm fibulin-3's pro-invasive role in bladder cancer and to delineate the mechanisms involved, as described in the next chapter.

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## Chapter III

## Introduction to Fibulin-3

In the previous chapter, fibulin-3 was found to be highly expressed in the MIBC compared to NMIBC and its expression was positively correlated with the invasion and migration of bladder cancer cell. Since our experimental results suggested fibulin-3 to play a potential role as a pro-invasive factor, a review of fibulin-3 is first presented here in this chapter.

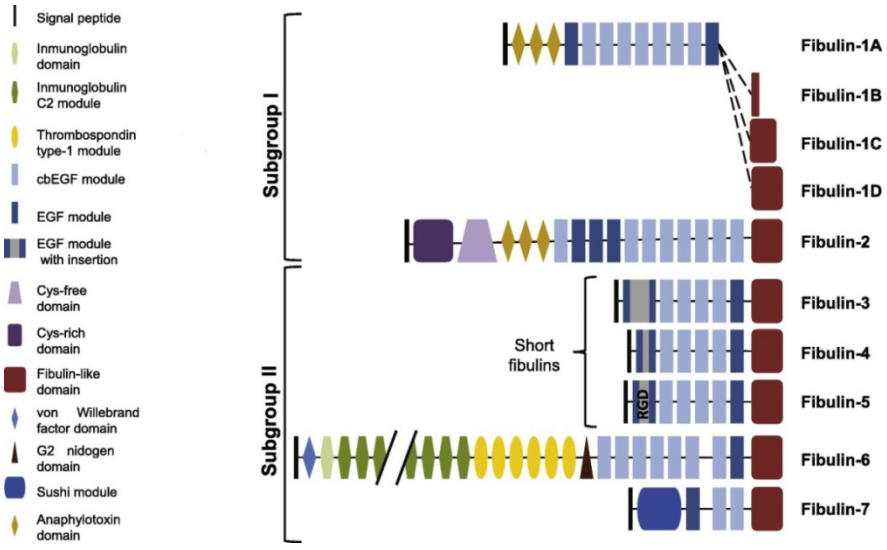


**Figure 3-Family of fibulins characterized by their tandem arrays of epidermal growth-factor-like domains (cbEGF) and a distinct C-terminal fibulin-like domain.**
Structural component of each fibulin member with the alternative spliced forms of fibulin-1. Fibulin-5 is the only member that contains an integrin binding interaction sequence, RGD: arginine-glycine-aspartic acid. Adapted from The dual role of fibulins in tumorigenesis, Obaya AJ, Rua S, Moncada-Pazos A, Cal S. 325:132-8, Copyright (2012), with permission from Elsevier (1).

**Fibulins**

Fibulins are a family of secreted extracellular matrix glycoproteins. They are characterized by tandem arrays of epidermal growth factor-like domains and a distinct C-terminal fibulin-type module. They function as intramolecular bridges that stabilize the organization of extracellular matrix (ECM) structures, such as elastic fibers and basement membranes (2). Currently, there are seven known members in this family and they have been subdivided into 2 groups (Fig. 3). Fibulin-1 and fibulin-2 compose the first subgroup and are much larger than the second group's members (with the exception of fibulin-6) due to the presence of an extra domain with three anaphylatoxin modules and higher number of calcium binding epidermal group factor (cbEGF) modules. Fibulin-2 (200kDa) is larger than fibulin-1 (100kDa) as it contains an additional N-terminal domain of ~400 amino acids that is not found in any other member of its family. Fibulin-1 also has four alternative forms that differ in the carboxyl terminal region (Fig. 3). Fibulin-1 and -2 have by far been the most studied of all the fibulins. These two fibulins localize in the basement membranes, elastic fibers, and in other connective tissues and are widely expressed in the endocardial cushion tissue, great vessels, and developing cartilages during embryogenesis and remain abundant in the cardiac valves and blood vessel walls in the postnatal stage (3). They have been found to have distinct yet overlapping properties. However, both have been found to bind various proteins including fibronectin (4), proteoglycans (5), tropoelastin (6), and basement membrane proteins (7).

The rest of the fibulins fall into the second group: Fibulins 3-5 were originally identified as "short fibulins" (the main distinction for this group until the discovery of fibulin-6) as they are all fairly similar in size (50-60kDa) and have similar modular structure, but differ in their N-terminal-modified cbEGF size. Fibulin-7 is also of similar size with a molecular weight of

50kDa, but is distinct from the others by the presence of a module named “sushi” that is involved in protein-protein interactions. On the other hand, fibulin-6 is the largest member of the fibulin family with a molecular weight of 615kDa that is attributed to 44 tandem immunoglobulin and 6 thrombospondins type-I modules. Not much is known about this fibulin subgroup, especially in comparison with the other subgroup. However, studies of these fibulins, particularly in their involvement in human diseases, are starting to reveal their unique properties and features.

**Fibulin-3: Structure and Expression**

Fibulin-3, also known as EGF-containing fibulin-like extracellular matrix protein 1, (EFEMP1), is encoded by the EFEMP1 gene that is located on chromosome 2p16. It contains 12 exons and consists of 493 amino acids with a molecular weight of 55kDa. Fibulin-3 is highly conserved amongst human, rat, and mouse (92-94% identical amino acid sequence). Structurally, fibulin-3 consists of a signal peptide at the N-terminal domain, a modified cbEGF domain followed by five tandem arrays of cbEGF domains, and a C-terminal fibulin-type module. The modified cbEGF domain contains an 88 amino acid insert. Under physiological conditions, fibulin-3 exists as monomers. During development, fibulin-3 is expressed in the condensing mesenchyme, giving rise to bone and cartilaginous structures. In adults, fibulin-3 is widely distributed in various tissues, particularly highly expressed in the basement membranes of epithelial and endothelial cells (8).

**Fibulin-3: Function**

Fibulin-3 was originally identified as a gene that was highly upregulated in senescent and Werner syndrome fibroblast (9). A missense mutation in the fibulin-3 gene (R345W) causes Malattia Leventinese (ML) (also known as Doyne’s honeycomb retinal dystrophy), an inherited autosomal dominant macular degenerative disease. It is characterized by yellow-white deposits,

drusen that accumulate beneath the retinal pigment epithelium (RPE). The phenotype displayed of ML patients is similar to that of age-related macular degeneration, which accounts for ~50% of registered blindness in the Western world. It was found that mutant fibulin-3 is misfolded and thus secreted inefficiently and retained inside the retinal pigment epithelial cells, although it did not appear to be a major component of drusen (10). Fibulin-3 knockout mice display reduced reproductive ability and have an early onset of aging, which include reduced lifespan, decreased body mass, lordokyphosis, reduced hair growth, and generalized fat, muscle and organ atrophy. The fine elastic fibers were reduced and disrupted in fascia, adventitia, small blood vessel walls, and vaginal walls. These mice also developed multiple hernias consisting of inguinal hernias, pelvic prolapse, and protrusions of the xiphoid process; however, they did not show signs of macular degeneration indicating that the loss of fibulin-3 function does not likely contribute to this aspect of aging (11).

Fibulin-3's role as an extracellular matrix glycoprotein is thought to also modulate cellular behavior and function by binding and integrating multiple molecules in the ECM. Some of these various binding partners include basement membrane protein extracellular matrix protein 1 (ECM1) (12), tissue inhibitor of metalloproteinase-3 (TIMP3) (13), hepatitis B virus-encoded X antigen (14), and relatively weak binding to elastin monomer tropoelastin (3). The integrity of the basement membrane is likely modulated by these interactions by serving as anchors to different ECM components. Not only does fibulin-3 function as a structural ECM component, but studies have reported it as a modulator for various cellular processes, such as cell growth, differentiation, angiogenesis, and tumor growth (15).

**Fibulin-3 and Cancer**

The role of fibulin-3 in cancer is unclear as there are several contradictory reports. Fibulin-3 may have a dual role either as a tumor suppressor or tumor promoter based on cancer cell context, which could account for the contradictory reports. For instance, fibulin-3 has been shown to elicit a tumor suppressive effect in breast (16), colorectal (17, 18), hepatocellular (19, 20), lung (21, 22, 23), nasopharyngeal (24), and prostate cancer (25). Inactivation of the fibulin-3 promoter due to hypermethylation appears to account for the downregulation of fibulin-3 expression in these cancers. This downregulation has also been correlated with poor disease-free survival. For example, fibulin-3 gene methylation is an indicator of poor prognosis in colorectal cancer (17, 18). Although fibulin-3 has been shown to behave as a tumor suppressor, the underlying mechanism of its action remains uncertain.

On the other hand, mechanistic roles for fibulin-3 in cancer are being identified. For instance, secreted fibulin-3 in the breast cancer microenvironment inhibits TGF-β signaling by interacting with the type I TGF-β receptor in both breast cancer and endothelial cells (16). Loss of fibulin-3 expression promoted TGF-β-induced epithelial-to-mesenchymal transition (EMT), migration, invasion, and endothelial permeability, whereas restoring its expression had inhibited these TGF-β-mediated effects (16). In nasopharyngeal carcinomas, not only is fibulin-3 downregulated and associated with advanced tumor stages and poor 5-year survival rate, but fibulin-3 expression can suppress cell migration and invasion by decreasing phospho-AKT activity (24). Overexpression of fibulin-3 also suppressed invasion and migration of lung adenocarcinoma cells and additionally decreased the expression of EMT activators, N-cadherin and Snail. Cell stemness, as examined by decrease spheroid formation and lower stemness markers (i.e. Sox2 and β-catenin), was also negatively regulated by fibulin-3 expression. This

was modulated by glycogen synthase kinase-3β (GSK3β)/β-catenin pathway and the upstream regulators of GSK3β, including phosphoinositide 3-kinase (PI3K)/AKT and insulin-like growth factor-1 receptor (IGF-1R) (22). Wnt/β-catenin signaling in relation to fibulin-3's role in invasion and metastasis was also described in lung cancer, where fibulin-3 inhibited extracellular signal-regulated kinase (ERK) to activate GSK3β and inhibited MMP7 expression, which is induced by Wnt/β-catenin signaling (21). Other findings have indicated fibulin-3 to attenuate the invasiveness of lung cancer cells through p38-MAPK activation and decreased MMP-2/9 expression (23). Another relationship between p38α MAPK, fibulin-3 expression, and invasion/migration was presented in a study in mouse embryonic fibroblasts (MEFs). p38α downregulated fibulin-3 expression through hypermethylation of CpG sites, which was mediated by p38α stabilization of DNA methylase, *dnmt3a* mRNA (26).

In contrast to these tumor suppressor activities of fibulin-3, Arechederra M et al. demonstrated that fibulin-3 promotes migration and invasion *in vitro* through p38α activation, which also induced tumor growth in HCT116 (human colon cancer) cells (26). It was proposed that there may be somewhat of a negative feedback loop that exists as p38α simultaneously limits fibulin-3 expression. Furthermore, fibulin-3 has been found to have oncogenic activity in malignant gliomas, ovarian carcinoma, cervical carcinoma, and pancreatic carcinoma. Fibulin-3 expression was upregulated in ovarian carcinoma and its overexpression was significantly associated with high stage, low differentiation, lymph node metastasis, and poor prognosis (27). Additionally, fibulin-3 expression had positively correlated with microvascular density (MVD) and vascular endothelial growth factor (VEGF). Similarly, this was also found in cervical carcinoma where fibulin-3 expression had positively correlated with MVD and *VEGF* mRNA as well as lymph node metastasis, vascular invasion, and poor survival (28). Song EL et al. also

took this correlation further by investigating the role of fibulin-3 in angiogenesis and growth of cervical cancer *in vivo* with fibulin-3 overexpression promoting an increased growth rate, VEGF expression, and MVD (29). Besides fibulin-3's potential role in angiogenesis and vascularization, it was found to bind the epidermal growth factor receptor (EGFR) and activate MAPK and AKT pathways in pancreatic carcinoma (30). Conversely, fibulin-3 was reported to actually repress EMT, decrease aldehyde dehydrogenase 1 (ALDH1) isozymes and cancer stemness through c-met activation (31), suggesting an alternative role of fibulin-3 in pancreatic cancer.

Multiple efforts have been made in delineating fibulin-3's role in glioma (32, 33, 34). It was found that fibulin-3 was highly upregulated in gliomas and cultured glioma cells and absent in normal brain (32). Overexpressing and knocking down fibulin-3 expression did not seem to affect glioma cell morphology or proliferation; instead, its expression had enhanced substrate-specific cell adhesion and promoted cell motility. Elevated expression and activity of matrix metalloproteases, particularly MMP-2/MMP-9 and ADAMTS-5 were also seen with fibulin-3 overexpression (32). In addition, fibulin-3 was found to be a novel soluble activator of Notch signaling that antagonized DLL3, promoting tumor cell invasion in a Notch-dependent manner (33). Conversely, downregulating fibulin-3 had resulted in increased apoptosis, reduced self-renewal of glioblastoma-initiating cells, and impaired growth and dispersion of intracranial tumors. Further studies revealed fibulin-3 to be a paracrine activator of Notch signaling in endothelial cells, thus promoting angiogenesis (34) as fibulin-3 overexpression increased tumor VEGF levels, microvascular density, and vessel permeability, similar to what was observed in cervical (29) and ovarian cancer (27). Fibulin-3 also increased ADAM10/17 activity in endothelial cells by inhibiting the metalloprotease inhibitor TIMP3, which increased Notch

cleavage and DLL4 expression (34). Taken together, these reports indicate that fibulin-3 has multiple roles in cancer, but whether it is tumor promoting or tumor suppressing appears to depend on cell context.

**Summary-**

Fibulins are a group of extracellular matrix glycoproteins characterized by tandem arrays of epidermal growth factor-like domains and a C-terminal fibulin-type module. They are widely expressed, modulating and stabilizing the ECM, and commonly associated with elastic tissues and vasculature. Among this family, a short fibulin, fibulin-3 has been emerging as a pro- or anti-tumorigenic factor based on tissue cell context. The exact role it plays in cancer remains uncertain; however, it has been demonstrated that outside its role as a secreted glycoprotein, fibulin-3 is involved with vastly different roles that include regulating matrix metalloproteases, angiogenesis, EMT, and signaling pathways. These activities all contribute to tumor development and progression suggesting a potentially important role of fibuin-3 in regulating cancer progression/suppression. Cell context appears to define whether fibulin has tumor suppressing or tumor activating properties. Further investigation of fibulin-3 may lead to defining additional mechanisms of fibulin-3's role in cancer that can help translate its potential relevance into the clinic.

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## Chapter IV

## Role of fibulin-3 in regulating bladder cancer invasion

**Abstract-**

As described in chapter II, we previously discovered fibulin-3 to be highly expressed in the MIBC compared to the NMIBC. In addition, fibulin-3 expression was correlated with the invasion of different bladder cancer cells. To determine a functional role for fibulin-3 in bladder cancer invasion, here we modulated fibulin-3 expression in bladder cancer cell lines using lentiviral transduction. Knockdown of fibulin-3 expression inhibited the invasion and migration, while fibulin-3 overexpression promoted the invasiveness of bladder cancer cell lines. Our *in vivo* studies involving orthotopic injection of the fibulin-3 knockdown bladder cancer cells also showed a decrease in muscle invasive bladder tumor incidence compared to control cells. Furthermore, we identified insulin-like growth factor protein 5 (IGFBP5) as downregulated upon fibulin-3 knockdown and overexpressing IGFBP5 in these cells had restored the invasive and migratory potential that was inhibited by the knockdown of fibulin-3 expression. Taken together, these results suggest that fibulin-3 and IGFBP5 expression may contribute to the progression and invasion of MIBC and could represent potential biomarkers or therapeutic targets for improved bladder cancer diagnosis and/or treatment.

**Introduction-**

Fibulin-3 (FBLN3, EFEMP1) is an extracellular matrix glycoprotein, characterized by the tandem calcium-binding epidermal growth factor-like repeats and a C-terminal fibulin-type

module. It is widely expressed throughout the body during development and in adult tissues, localizing in the condensing mesenchyme and the basement membrane of epithelial and endothelial cells, respectively (1). It has been reported to play a structural role in the extracellular matrix interacting with other basement membrane proteins (2, 3) as well as activate downstream signaling pathways, such as EGFR (4). Studies in cancer have revealed that fibulin-3 can behave as either a tumor suppressor or promoter based on cancer type. However, the exact roles fibulin-3 plays in these cancers are still relatively undetermined. In addition, there have not been previous reports about the relationship between fibuilin-3 and bladder cancer to our knowledge. With fibulin-3 appearing to be a novel candidate, we aimed to understand its potential role in bladder cancer progression. Since our earlier findings identified and confirmed differential expression of fibulin-3 between MIBC and NMIBC, here we sought to determine whether modulating fibulin-3 expression can affect the invasion and migration of bladder cancer cells both *in vitro* and *in vivo* as well as investigate possible mechanisms involved.

**Materials and Methods-**

*Cell Lines*

T24, UMUC-13, UMUC-3, 5637, and RT4 cell lines were generously provided by Dr. Monica Liebert (University of Michigan, Ann Arbor). All cells, except RT4, were maintained in Dulbecco's Modified Eagle Medium, DMEM, high glucose (Gibco-Life Technologies (GIBCO), Carlsbad, CA) supplemented with 10% fetal bovine serum, 100U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% $CO_2$ at $37^0$C. RT4 cells were maintained in DMEM-F12 (GIBCO) complete medium. Cell lines were authenticated using short tandem repeats and were evaluated monthly to ensure they were Mycoplasma free.

*Transduction with shRNA, scrambled shRNA, empty vector, and cDNA vector*

pGFP-C-shLenti lentiviral plasmids (Origene, Rockville, MD) were used to stably transduce T24 and UMUC-13 cells with Fibulin-3 shRNA (shFBLN3 #1 or shFBLN3 #2 constructs) or scrambled control. Subsequent transient overexpression transductions used lenti ORF clones of human fibulin-3 or IGFBP5 along with the empty vector plasmid (Origene). Briefly, all lentiviral particles for the transductions were generated through transfection with HEK293T cells using the lenti-v-pak packaging kit (Origene). Lentiviral particles were collected from the HEK293T cells in 2 batches- 24 and 48 hours after changing medium 12-18 hours within the transfection. Lentiviral particles were added to the cells with addition of 8mg/ml of polybrene (Sigma, St. Louis, MO). Transduction medium was removed 24 hours after addition and replaced with fresh growth medium. Cells were incubated for 2 more days before adding puromycin (InvivoGen, San Diego, CA) at a concentration of 1μg/ml to establish stable fibulin-3 knockdown cells.

*RNA Extraction/qRT-PCR/RT$^2$ Profiler Array*

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) and purified with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA concentrations were measured using a Nanodrop Spectrophotometer ND-1000 (Wilmington, DE). 1 μg of total RNA was used for reverse transcription using SuperScript III First-Strand Synthesis System (Invitrogen). qRT-PCR was performed in triplicates using SYBR Green (Qiagen) with the LightCycler 480 instrument (Roche, Indianapolis, IN). Primers were chosen using Primer-BLAST and ordered from Invitrogen (Table 3.1).

For the Human Cancer Pathway RT$^2$ Profiler PCR Array (Qiagen), 400ng of total RNA was used for reverse transcription using RT$^2$ First Strand kit (Qiagen) according to the

manufacturer's protocol and qRT-PCR was performed using SYBR Green with the LightCycler 480 instrument (Roche). $C_T$ values were analyzed using the GeneGlobe Data Analysis Center (Qiagen).

*Western Blotting*

Cells were lysed with RIPA buffer complemented with protease inhibitor cocktail, PMSF, NaF, and $Na_3VO_4$, collected into a 1.5ml Eppendorf tube, and placed on ice and vortexed intermittently for 20 minutes. After centrifugation at 15,000rpm for 15 minutes at $4^0$C, supernatant was collected and measured for protein concentration using Pierce™ BCA Protein Assay (Thermo Fisher Scientific, Carlsbad, CA). 30-50μg of protein was resolved in a 4-20% gradient Novex™ Tris-Glycine gel (Thermo Fisher Scientific) under reducing conditions and transferred onto an Immun-Blot PVDF membrane (BioRad, Hercules, CA). Appropriate primary antibody was added after blocking for an hour with 5% non-fat milk: anti-fibulin-3 (TA503772, Origene), anti-IGFBP5 (55205-1-AP, Proteintech, Chicago, IL), and anti-β-actin (A5441, Sigma). The antigen-antibody reaction was detected using the appropriate horseradish peroxidase-conjugated secondary antibody, followed by visualization with the electrochemiluminescence detection system using film.

*Cell Growth*

Parental and transduced bladder cancer cells were plated in triplicates in 6-well plates at a density of $1.0\text{-}1.5x10^5$cells/well and incubated for either 24, 48, or 72 hours. At the indicated time points, cells were trypsinized, collected, and counted using the TC10™ automated cell counter (BioRad).

*Invasion and Migration Assay*

Cell invasion and migration was determined *in vitro* using transwell inserts with 8.0-μm pore size; invasion wells were coated with Matrigel (Corning BioCoat Chambers, Corning, NY). Cells were plated in 100-mm culture dishes and grown to 70-80% confluence. Cells were washed with 1X PBS and then trypsinized. After washing with 1X PBS twice, cell number was determined for 1-2 x $10^5$ cells/ml cell suspension in serum free medium. 500μl of the cell suspension was added to the upper chamber of the transwell (triplicates) and 10% FBS complete medium served as the chemoattractant in the lower chamber. After 24 hours of incubation, cells were gently removed from the upper well of the chamber using cotton-swaps and the cells that invaded through the membrane were stained with PROTOCOL Hema3 staining system (Fisher Scientific, Hampton, NH). Stained transwells were left to dry overnight and mounted on glass slides the next day and were counted using a light microscope. In most cases, five fields (40X) were counted for each transwell and the mean number of cells that invaded per field was calculated. However, for the 5637 and RT4 cells, the total number of cells that invaded/migrated were counted due to their low invasive/migratory nature.

*Orthotopic Bladder Injections*

Three to four month old male NOD-SCID mice were anesthetized with isoflurane and administered Rimadyl®-(carprofen) (Pfizer Animal Health, New York, NY) (5mg/kg) i.p prior to injection. A small longitudinal incision was made in the lower abdomen to identify the bladder. Then 1.5 x $10^6$ T24 scramble/shFBLN3#1/shFBLN3#2 cells or 5 x $10^6$cells UMUC-13 scramble/shFBLN3#1 cells in a 50μl suspension in DMEM were injected into the lumen of the bladder. 10 mice were injected per cell line. After injection, the peritoneum was sutured with coated VICRYL 5-0 absorbable suture (Ethicon, Somerville, NJ) and the skin was closed up

using wound clips. Mice were monitored throughout the entire surgical process and daily until the clips were removed (10 days), and once a week for tumor monitoring thereafter. Mice were sacrificed 4 weeks after being injected. Bladders were resected and placed into tissue cassettes and kept in formalin for 48 hours. Tissues were then washed three times with PBS and placed in 70% ethanol for paraffin embedding and for H&E staining. Sections were evaluated by a board-certified anatomic pathologist with subspecialty experience in genitourinary pathology (Dr. Scott Tomlins) for the presence of tumor and depth of invasion (if present).

*Statistical Analysis*

Experiments were repeated two to three times and statistically analyzed using GraphPad Prism 6. Multiple groups were compared using one-way ANOVA, whereas experiments involving two groups were analyzed using student t-test. Pearson correlation was used to determine correlation between two variables and Chi-square was performed to determine the statistical significance with the *in vivo* studies. p-values that were less than 0.05 were considered significant.

| Gene | Primer Sequence (5'-3') |
|---|---|
| Fibulin-3 | F: TGA GCT AAG CAG TGA CAG GC<br>R: GTA TCC CTG GGG GCA CAT AC |
| IGFBP5 | F: AAG ATC GAG AGA GAC TCC CGT<br>R: TCT GCG GTC CTT CTT CAC TG |
| β-actin | F: GAT GAG ATT GGC ATG GCT TT<br>R: CAC CTT CAC CGT TCC AGT TT |

**Table 4.1-List of primers used in qRT-PCR experiments.**

Primers were designed using Primer-Blast.

**Results-**

*Fibulin-3 knockdown inhibits bladder cancer cell invasion and migration*

To study the role of fibulin-3 in bladder cancer, stable fibulin-3 knockdown T24 and UMUC-13 cells lines were created, which were repeatedly validated for fibulin-3 expression throughout use in the study using qRT-PCR and western blotting. Fibulin-3 transcript and protein expression were consistently knocked down (70-80%) in the shFBLN3 #1 and shFBLN3 #2 compared to scrambled shRNA control in both the T24 and UMUC-13 cells (Fig. 4.1A). Lentiviral transductions with the fibulin-3 or scrambled shRNAs were not associated with any differences in the cells' morphology (data not shown) nor changes in the cells' growth rate as compared to their parental controls (Fig. 4.1B). We next evaluated if modulation of fibulin-3 expression altered the cells' invasive ability. We found that knockdown of fibulin-3 expression inhibited both the invasive and migratory capability of both the T24 and UMUC-13 cell lines (Fig. 4.1C).

*Fibulin-3 overexpression promotes bladder cancer cell invasion and migration*

As decreasing fibulin-3 expression diminished invasion, we wanted to next determine whether fibulin-3 overexpression would confer an invasive phenotype in the low invasive and low fibulin-3 expressing 5637 and RT4 cells. Transiently transduced 5637 and RT4 fibulin-3 overexpressing cell lines were produced (Fig. 4.2A). Similar to the fibulin-3 knockdown in the T24 and UMUC-13 cell lines, overexpression of fibulin-3 had no impact on cell morphology (data not shown) or cell growth rate (Fig. 4.2B). On the other hand, fibulin-3 overexpression promoted invasion and migration of the 5637 and RT4 cells compared to their E.V. controls (Fig. 4.2C). Taken together, these results demonstrate that fibulin-3 confers pro-invasive activity in bladder cancer cells.

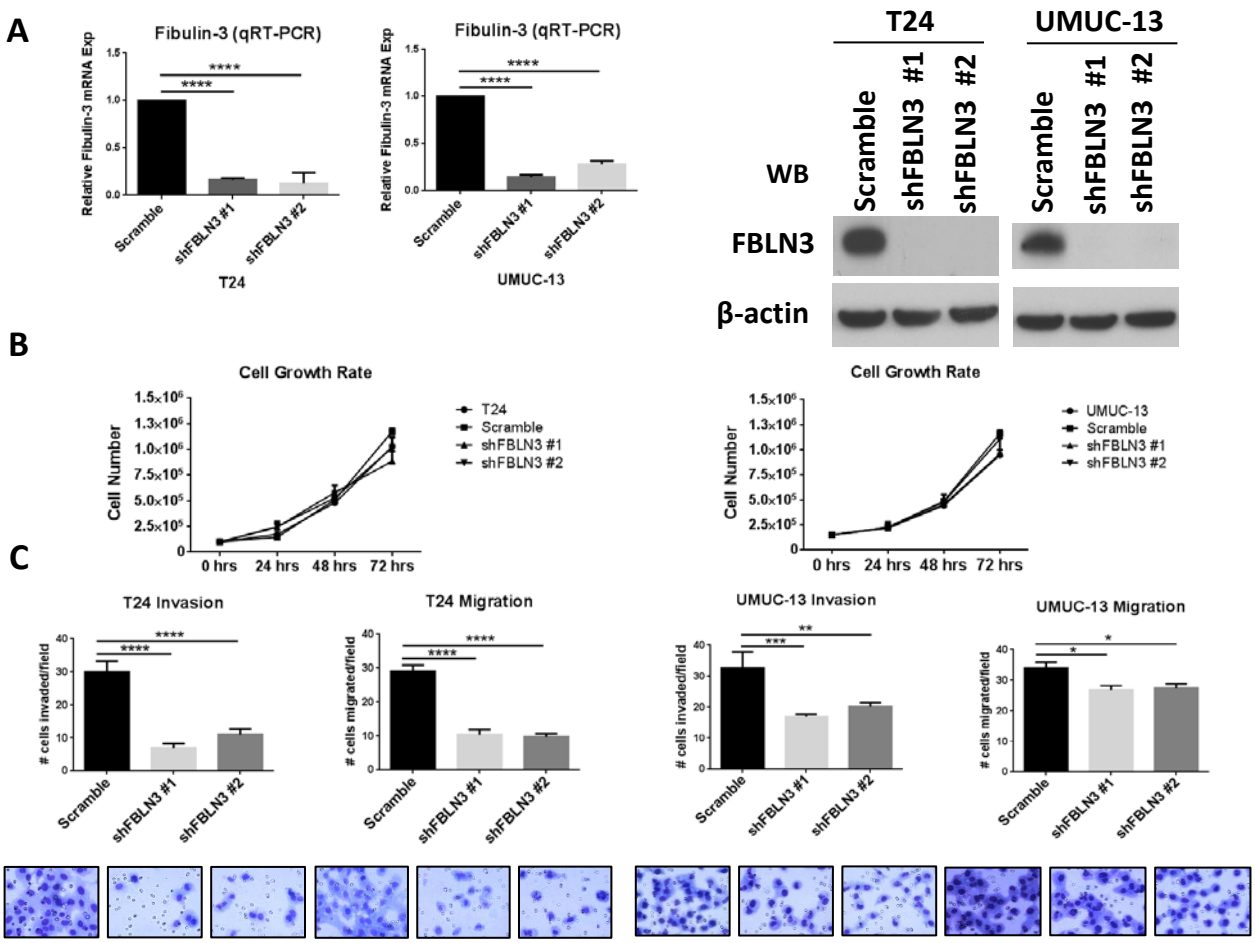


**Figure 4.1-Decreased fibulin-3 expression attenuates bladder cancer invasion and migration.**

T24 and UMUC-13 bladder cancer cells were transduced with fibulin-3 shRNA (shFBLN3 #1, shFBLN3 #2) or scrambled shRNA lentiviral particles. **A**) To measure mRNA, total RNA (1 μg) was used to make cDNA and then subjected to qRT-PCR using fibulin-3 and β-actin (loading control) primers. Fibulin-3 mRNA was normalized to β-actin. Results are shown relative to scrambled control as the means of three separate experiments; ****$p \leq 0.0001$, one-way ANOVA. To measure protein, total protein (30 μg) was loaded and subjected to western blotting. **B**) To evaluate cell growth, 1.0 x $10^5$cells/well (T24) and 1.5 x $10^5$cells/well (UMUC-13) were plated in 6-well plates in triplicates. Total cell numbers per well were counted 24, 48, and 72 hours after plating. **C**) To evaluate invasion and migration, T24 and UMUC-13 shFBLN3 knockdown and their respective scrambled control cells (5 x $10^4$cells/well) were plated in the upper well in serum-free DMEM of either Matrigel-coated or control transwell inserts to test for invasion and migration, respectively. Complete 10% FBS DMEM was placed in the bottom. After 24 hours, cells that invaded/migrated were fixed and stained. Invasive and migratory potentials were determined by counting the number of cells that invaded/migrated in five random 40X microscopic fields. The results are presented as the mean number of cells counted per field ± SD and representative images of the fields for each individual sample are displayed underneath (40X magnification). Results in C, *$p<0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$, by one-way ANOVA.

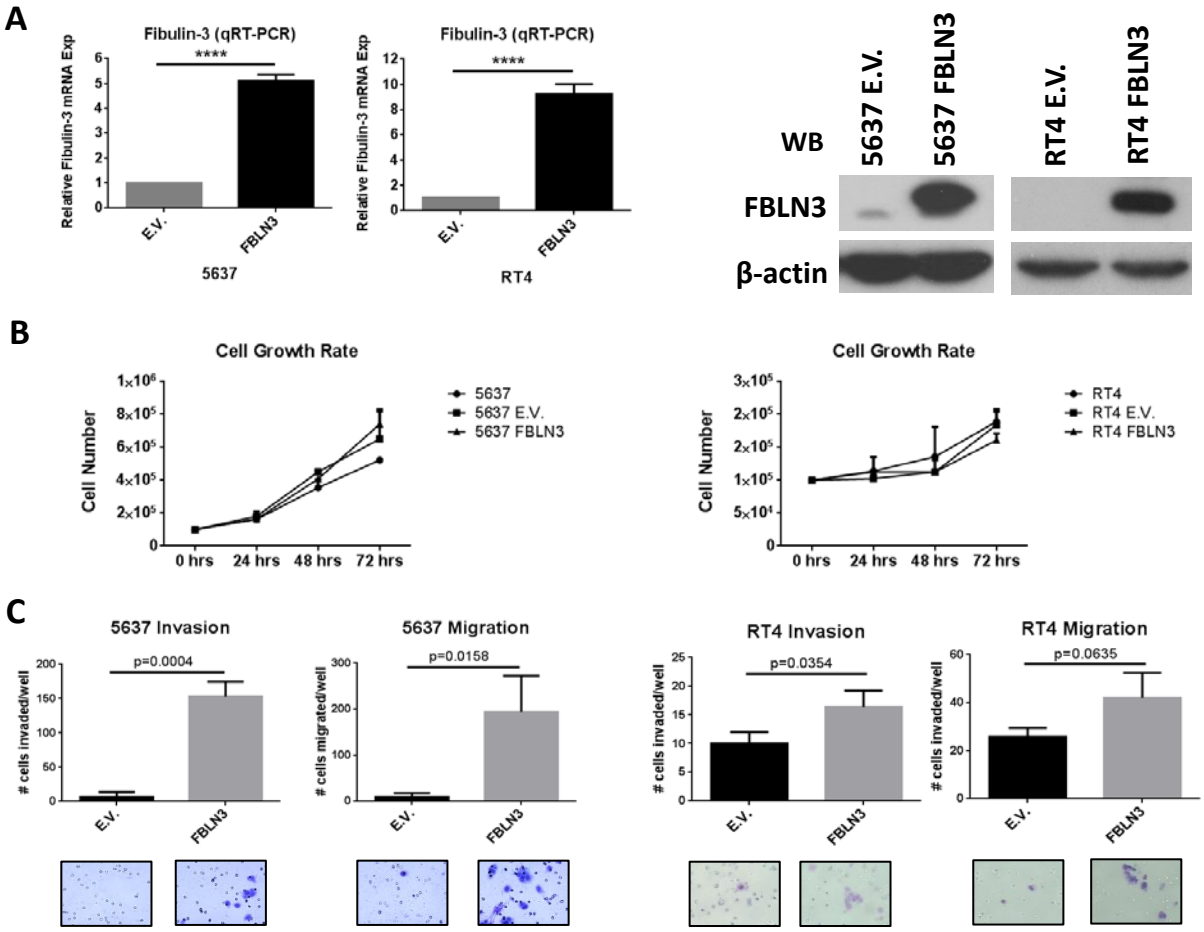


**Figure 4.2-Fibulin-3 overexpression promotes bladder cancer cell invasion and migration.**

Fibulin-3 was overexpressed in 5637 and RT4 bladder cancer cell lines through transduction with a lentiviral vector containing fibulin-3 cDNA or empty vector (E.V.). **A**) To measure mRNA, RNA (1μg) from the transduced cell lines was subjected to qRT-PCR using fibulin-3 and β-actin (loading control) primers. Fibulin-3 mRNA was normalized to β-actin and results are shown relative to E.V. as a means of three separate experiments; ****$p \leq 0.0001$, unpaired t-test. To measure protein, total protein (30 μg) lysates from the transduced cells were subjected to western blotting. **B**) To determine cell growth rates, cells were plated ($1.0 \times 10^5$ cells/well) in a 6-well plate (triplicates) and the total number of cells present after the indicated times were counted. Results are shown as mean ± SD. **C**) To evaluate invasion and migration, 5637- and RT4-transduced cells or empty vector (E.V.) controls-transduced cells were plated in the upper well ($1 \times 10^5$ cells/well) in serum-free medium of either Matrigel-coated or control transwell inserts to test for invasion and migration, respectively. Complete 10% FBS medium was placed in the bottom. After 24 hours, cells that invaded/migrated were fixed and stained. Invasive and migratory potentials were determined by counting the total number of cells that invaded/migrated. The results are presented as the total number of cells counted per transwell ± SD and representative images of the fields for each individual sample are displayed underneath (40X magnification); unpaired t-test.

*Fibulin-3 knockdown decreases muscle invasive bladder cancer in vivo*

Since fibulin-3 was revealed to play a role in bladder cancer invasion and migration *in vitro*, we wanted to test whether the same would translate *in vivo*. T24 scramble, shFBLN3 #1, and shFBLN3 #2 as well as UMUC-13 scramble, shFBLN3 #1 stably transduced cell lines were injected directly into the lumen of mice bladders. Mice (10/group) were euthanized 4 weeks after injection. Of the T24 scramble mice, which were injected on 2 separate occasions, 60% showed aggressive muscle invasive tumors (Fig. 4.3A, F), whereas only 20% of the T24 shFBLN3 #1 injected mice had T2 tumors and no tumors were found in the T24 shFBLN3 #2 injected mice (Fig. 4.3B, C, F). Of the mice that were injected with the UMUC-13 cells, 30% displayed muscle invasive tumors with the scramble cells and 10% in the shFBLN3#1 knockdown cells (Fig. 4.3D, E, F). Only the T24 scramble cells vs. shFBLN3 #1 and shFBLN3 #2 results were statistically significant ($p=0.0085$); however, in comparison to the scrambled shRNA control, UMUC-13 shFBLN3 #1 did have less incidence of muscle invasive bladder tumors.

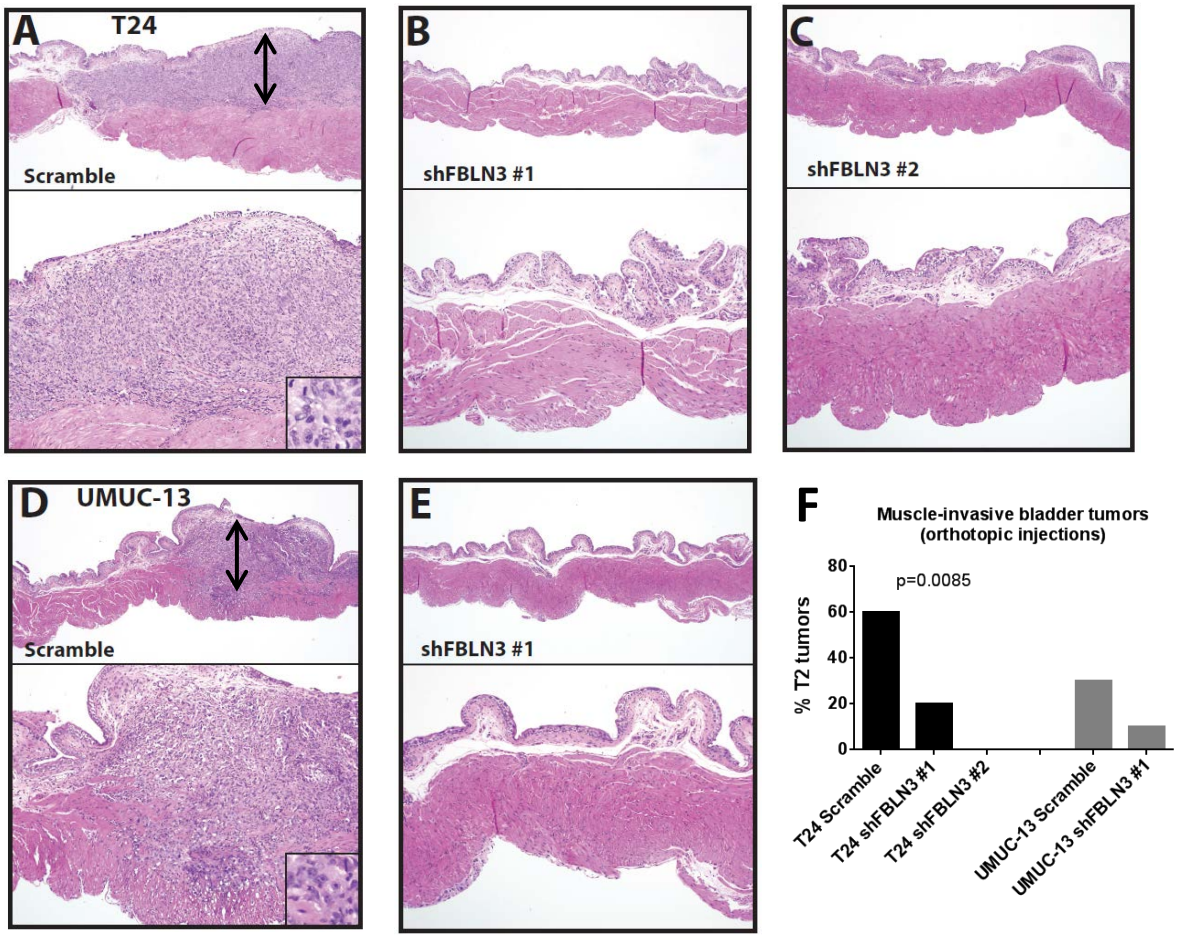


**Figure 4.3-Fibulin-3 knockdown decreases muscle invasive bladder cancer *in vivo*.**

The bladders of male NOD-SCID mice were surgically exposed and injected with either T24 scramble (**A**), shFBLN3 #1 (**B**), or shFBLN3 #2 (**C**) ($1.5x10^6$cells/50µl) cells through the bladder wall directly into the bladder lumen (n=10/group). Mice were euthanized 4 weeks after injection and bladder tissues were placed in formalin, followed by paraffin embedding. H&E stained slides were used to determine tumor formation/depth. Images of the bladder tissues were taken from a representative H&E stained slide of each group, magnification of 4X (upper box) and 10X (lower box) were used for the bladder images and 40X (inset) to display the cancer cells. Double arrow shows extent of tumor. UMUC-13 scramble (**D**) and shFBLN3 #1 (**E**) ($5x10^6$cells/50µl) cells were also injected orthotopically and mice were euthanized at 4 weeks as described above. **F**) Graph represents the percent of mice that displayed muscle invasive T2 bladder tumors in the different T24 and UMUC-13 groups (n=10/group); Chi-square analysis.

*Fibulin-3 knockdown results in the reduction of IGFBP5 expression*

To develop an understanding through which signaling pathway fibulin-3 may modulate invasion, we evaluated differential mRNA expression between fibulin-3 knockdown (shFBLN3 #1) versus fibulin-3 intact (scrambled control) cells for both the T24 and UMUC-13 cells using arrays consisting of genes associated with cancer pathways and invasion. Specifically, the Human Cancer PathwayFinder RT$^2$Profiler PCR Array was run on the Roche 480 LightCycler, leading us to insulin-like growth factor binding protein 3 (IGFBP3) and insulin-like growth factor binding protein 5 (IGFBP5). IGFBP3 and IGFBP5 expressions were downregulated in the fibulin-3 knockdown cells compared to control in both the T24 and UMUC-13 cells (Fig. 4.4A). To further support the array-based findings, we examined the Oncomine database for IGFBP3 and IGFBP5 expression in bladder cancers. Analysis using the Oncomine database revealed that there was higher expression of IGFBP5 in infiltrating bladder urothelial carcinomas and recurrent tumors, coinciding with the higher expression of fibulin-3 that was seen when we originally nominated the gene (Fig. 4.4B). In addition, fibulin-3 and IGFBP5 expression were analyzed with the published TCGA data from muscle invasive urothelial tumors (5) via cBioPortal (6, 7). A comparison between the two genes showed positive correlation, suggesting potential co-expression of fibulin-3 and IGFBP5 in MIBC (Fig. 4.4C). On the other hand, the same was not found to be true with IGFBP3, so we focused our efforts on investigating IGFBP5 and its possible relationship with fibulin-3 expression as it seemed more of a likely candidate. Thus, to validate the array-based findings in the cell lines, we first measured IGFBP5 mRNA and protein expression. Knockdown of fibulin-3 decreased IGFBP5 expression in both the T24 and UMUC-13 cells (Fig. 4.4D), which was consistent with the array and database findings.

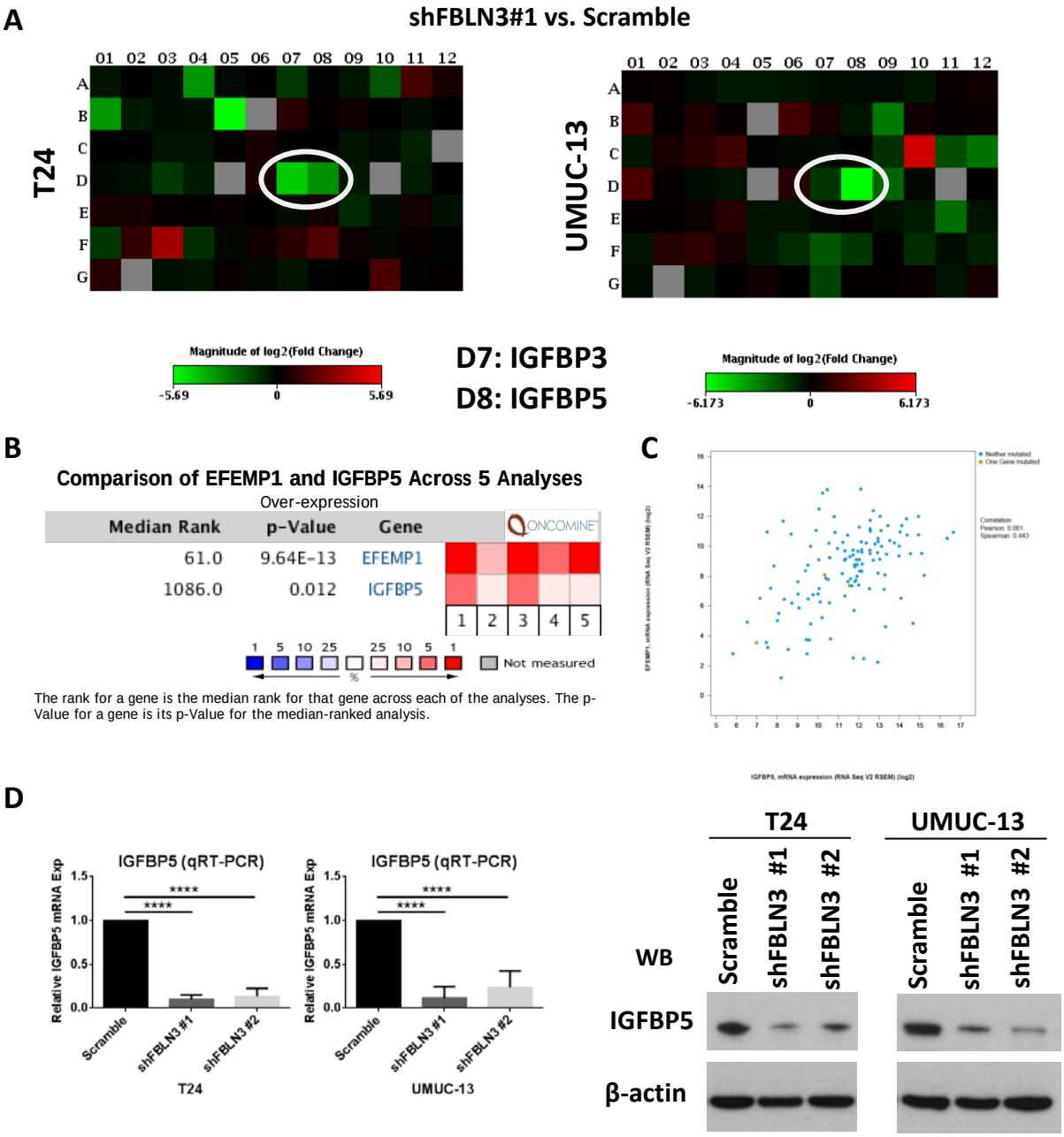


**Figure 4.4-IGFBP5 expression is downregulated with fibulin-3 knockdown.**

**A**) Total RNA was extracted from T24 shFBLN3 #1 and UMUC-13 shFBLN3 #1 cells and their respective scrambled shRNA cells and the RNA (400ng) used to generate cDNA was subjected to PCR using the Human Cancer PathwayFinder PCR array. $C_T$ values were analyzed using the GeneGlobe Data Analysis Center, which produced heat maps showing the difference in gene expression between the fibulin-3 knockdown and the scrambled control. IGFBP3 (location D7)

and IGFBP5 (location D8) are circled. **B**) The Oncomine gene database was explored for fibulin-3 (EFEMP1) and IGFBP5 expression across five different studies that looked at infiltrating bladder urothelial carcinoma as well as recurrence: **(1)**. Bladder Urothelial Carcinoma Type: Infiltrating Bladder Urothelial Carcinoma *Blaveri Bladder 2, Clin Cancer Res, 2005.* **(2)**. Bladder Urothelial Carcinoma Type: Infiltrating Bladder Urothelial Carcinoma *Dyrskjot Bladder 3, Cancer Res, 2004.* **(3)**. Bladder Urothelial Carcinoma – Recurrence *Lindgren Bladder, Oncogene, 2006.* **(4)**. Bladder Urothelial Carcinoma Type: Infiltrating Bladder Urothelial Carcinoma *Sanchez-Carbayo Bladder 2, J Clin Oncol, 2006.* **(5)**. Bladder Urothelial Carcinoma Type: Infiltrating Bladder Urothelial Carcinoma *Stransky Bladder, Nat Genet, 2006.* The meta-analysis showed both genes being over-expressed in infiltrating bladder urothelial carcinoma and recurrence, which were both highly significant. **C**) cBioPortal database was used to compare fibulin-3 and IGFBP5 expression against all the T2 bladder tumors from the TCGA study. Analysis indicated a correlation between the two genes (Pearson: 0.061, Spearman: 0.443), suggesting potential co-expression of fibulin-3 and IGFBP5 in MIBC. **D**) To measure mRNA, total RNA was extracted from the fibulin-3 knockdown and scrambled T24 and UMUC-13 cell lines. Total RNA (1 μg) was subjected to qRT-PCR using primers for human IGFBP5 and β-actin (as loading control); ****$p \leq 0.0001$, one-way ANOVA. To measure protein expression, protein lysates (50μg) were subjected to western analysis. Blots were probed with human IGFBP5 antibody and human β-actin antibody served as loading control.

*IGFBP5 expression restores the invasive and migratory potential inhibited by fibulin-3 knockdown*

To examine whether IGFBP5 plays a role in the invasive and migratory-modulating ability of fibulin-3, IGFBP5 was overexpressed in the T24 and UMUC-13 fibulin-3 knockdown cells. Transduced cells were first verified for IGFBP5 expression, which revealed efficient mRNA and protein overexpression (Fig. 4.5A). These cells were then evaluated for their invasive and migratory ability. Overexpression of IGFBP5 rescued the invasive ability that was lost by knockdown of fibulin-3 in both cell lines (Fig. 4.5B). To determine if the increased invasion

observed with the overexpression of fibulin-3 in the low invasive cells lines (from Fig. 4.2C) was associated with altered IGFBP5 expression, we measure their levels in the fibulin-3 overexpressing lines. Fibulin-3 overexpression increased IGFBP5 expression in both the 5637 and RT4 cell lines (Fig. 4.5C), suggesting a direct correlation between fibulin-3 and IGFBP5 expression as well as invasion.

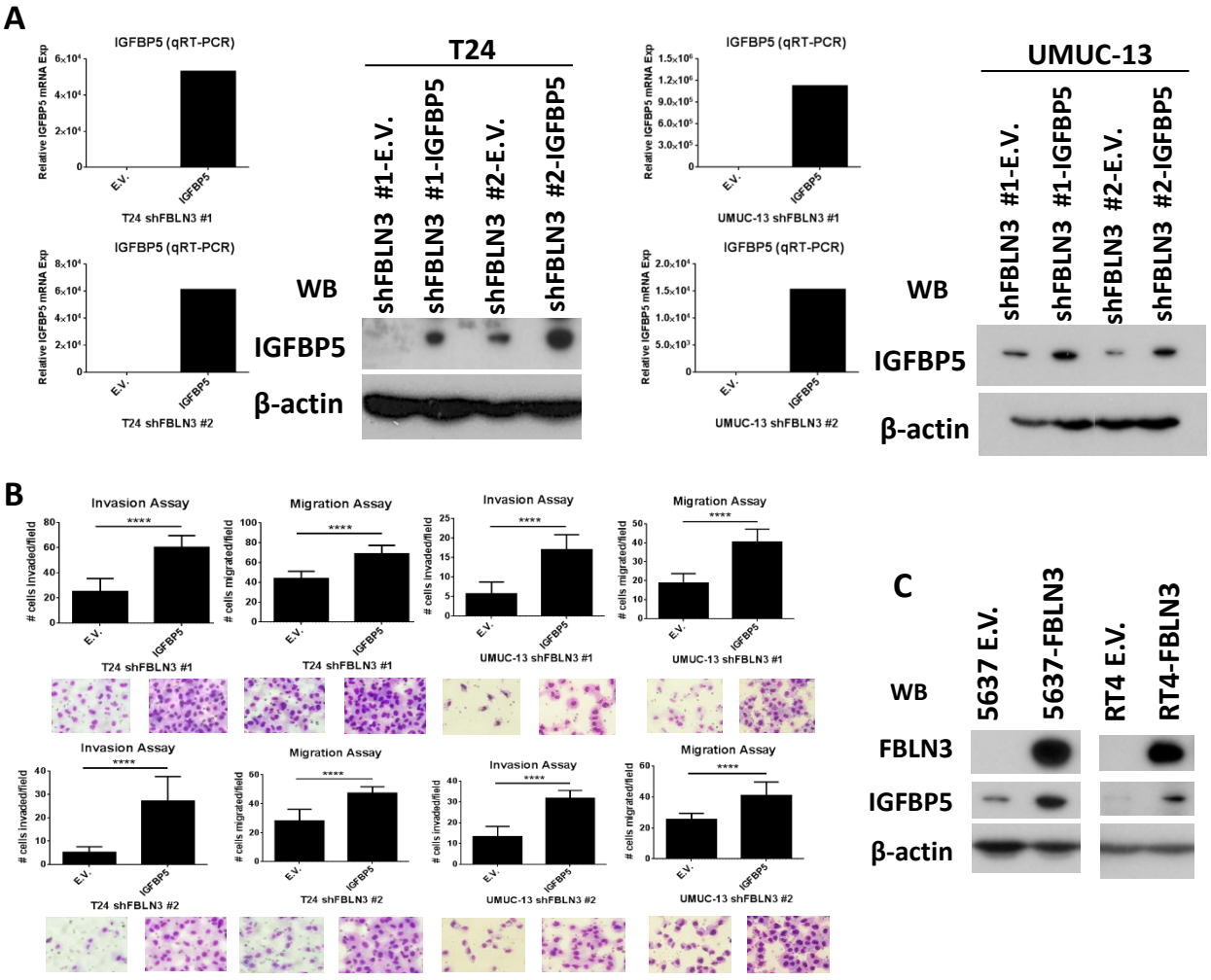


**Figure 4.5-IGFBP5 rescues the invasive and migratory potential that is decreased through loss of fibulin-3 expression. A**) IGFBP5 was overexpressed in the fibulin-3 knockdown T24 and UMUC-13 cells through lentiviral transduction with IGFBP5 cDNA or empty vector (E.V.). Overexpression was validated using qRT-PCR and western blotting. Total RNA extracted from the transduced cells (1 μg) was subjected to qRT-PCR using primers for human IGFBP5 and β-

actin (as loading control); one-way ANOVA. To measure protein expression, protein lysates (50μg) were subjected to western analysis. Blots were probed with human IGFBP5 antibody and human β-actin antibody served as loading control. **B**) IGFBP5 overexpressed T24 shFBLN3 #1, T24 shFBLN3 #2, UMUC-13 shFBLN3 #1, UMUC-13 shFBLN3 #2 as well as their respective E.V. controls were subjected to invasion and migration transwell assays. 5 x $10^4$ cells were plated in the upper transwell and incubated for 24 hours at which time cells were fixed and stained for counting. Results are shown as the mean number of cells counted per five random 40X microscopic fields ± SD and images of a representative 40X field per sample are shown below; ****$p \leq 0.0001$, by unpaired t-test. **C**) Protein lysates (50μg) from fibulin-3 overexpressing 5637 and RT4 cells with their E.V. controls were subjected to western blotting for IGFBP5 protein expression with β-actin serving as loading control.

**Discussion-**

Compared to the well-studied fibulin-1 and fibulin-2 of the fibulin family, much is still unknown about fibulin-3 and the exact roles it plays biologically. However, studies have started unraveling the mysteries of this glycoprotein, especially in relation to cancer. Depending on tissue context, papers have shown fibulin-3 to be involved as a tumor promoter or tumor suppressor. For instance, fibulin-3 has been reported to be overexpressed and associated with tumor progression and poor prognosis in glioma (8), pancreatic (9), cervical (10), and ovarian carcinomas (11) by promoting cell survival and invasion. In contrast, the opposite is seen in breast (12, 13), lung (14, 15, 16), colorectal (17, 18), prostate (19), and nasopharyngeal carcinomas (20). In these cases, fibulin-3 was reported to be downregulated due to hypermethylation of its promoter, which correlated with cancer progression and poor prognosis. The mechanisms through which fibulin-3 is regulating these different and opposite roles is still uncertain, but studies are beginning to uncover them as being involved with vastly different roles that include regulating matrix metalloproteases (MMPs) (8, 15, 21), angiogenesis (22, 23),

epithelial-to-mesenchymal transition (13, 14), and signaling pathways such as MAPK (24), AKT (4, 20), and Notch (22, 25).

We are unaware of any reports of fibulin-3 and the role it may play in bladder cancer. Our studies had found higher fibulin-3 expression in muscle invasive vs. non-muscle invasive patient bladder tumors and that its expression correlated with the invasive potential of bladder cancer cells, suggesting a pro-invasive role of fibulin-3 in bladder cancer. Herein, we were able to generate stable fibulin-3 knockdown cell lines in the T24 and UMUC-13 cells and transiently transduced 5637 and RT4 fibulin-3 overexpressing cells. Manipulation of fibulin-3 expression did not reveal differences in cell morphology nor promote cell proliferation, which was the case for glioma cells where fibulin-3 was shown to be oncogenic (8). On the other hand, our invasion and migration assays showed fibulin-3 to be a pro-invasive factor as knockdown of fibulin-3 inhibited and overexpression of fibulin-3 promoted invasion and migration. Hu et al. had also reported enhanced cell motility, cell migration, and increase in tumor invasion in glioma cells through increased expression of matrix metalloproteases (MMPs). Contrary to the association of fibulin-3 and MMPs in glioma cells, we did not see any consistent changes with MMP expression with the fibulin-3 knockdown in bladder cancer cells when evaluated with gelatin zymography and MMP ELISAs (data not shown). These results indicate that in bladder cancer cells, fibulin-3 promotes invasion independent of modulating MMP levels.

The *in vivo* studies with fibulin-3 knockdown cells were consistent with the *in vitro* studies. Namely, that there was less muscle invasive bladder tumor incidence with the fibulin-3 knockdown cells compared to the scrambled controls. Furthermore, we did not observe differences in cell viability or cell growth rates between fibulin-3 knockdowns and scrambled control *in vitro*, so it is unlikely the difference in the incidence of muscle invasive tumors *in vivo*

is attributed to cell viability or growth rates. These findings support that fibulin-3 acts as a pro-invasive factor for muscle invasion. We did observe two muscle invasive tumors with the T24 shFBLN3 #1 mice and one with the UMUC-13 shFBLN3 #1, which indicates that fibulin-3 knockdown alone may not completely abolish the onset of muscle invasive bladder cancer though it can decrease the incidence. It is possible that this result occurred as although we achieved a significant reduction in fibulin-3 expression, it was not a complete knockout of this gene. The tumor take rate for the UMUC-13 cells were relatively low compared to the T24 cells, which did not allow us to observe a statistically significant difference in MIBC incidence with the UMUC-13 cells. The UMUC-13 cell line was derived from a lymphatic metastasis of TCC, while the T24 cell line came from high grade TCCs, potentially explaining the difference in tumor intake. One particular genetic difference is in their p53 expression: T24 cells were found to have low levels of p53 due to a p53 mutant resulting from an in-frame deletion of tyrosine 126 (24) whereas the UMUC-13 cells were found to have high levels of p53 (27). High grade muscle invasive tumors have been associated with loss of p53 function (28), so it is possible that this could influence the difference in tumor establishment rate between these two cell lines. One limitation of this model is that it could be challenging to identify actual T1 tumors due to the thin layer of lamina propria of the bladder in mice, especially in comparison to human. Thus, to be consistent in our analysis, the mice tumors were identified as either T2s or T0s. Nevertheless, a significant decrease in muscle invasive tumors was observed with the fibulin-3 knockdown specifically with the high grade T24 TCC cells, supporting fibulin-3's likely involvement in T2 invasion.

In order to identify mechanisms downstream of fibulin-3 we subjected the fibulin-3 knockdown and scrambled control bladder cancer cells to the Human Cancer PathwayFinder $RT^2$

Profiler Array analysis. This led to the identification that downregulation of fibulin-3 expression was associated with downregulation of both IGFBP3 and IGFBP5 expression. Additional analysis with Oncomine and cBioPortal revealed IGFBP5 to be a top candidate involved with fibulin-3 expression. The Oncomine database search had displayed higher IGFBP5 and fibulin-3 expression in the infiltrating urothelial carcinomas and recurrent tumors that was highly significant. Likewise cBioPortal showed a positive correlation between fibulin-3 and IGFBP5 expression, suggesting that these two genes may be co-expressed in advanced tumors. Analysis with IGFBP3 and fibulin-3 did not turn up similar results; thus, we focused primarily on IGFBP5 and fibulin-3.

IGFBPs are a family of secreted proteins that bind insulin-like growth factors-1 and -2 (IGF-1, IGF-2) with high affinity. They serve as transport proteins and modulate the biological actions of IGFs by altering the interactions of IGFs with their cell surface receptors. This could either involve inhibiting IGF action by preventing binding to IGF-1R or promoting IGF action through assisted delivery to the receptor. IGFBP5 is the most conserved out of its 6 member family and has been found to have a multifunctional role that can either be IGF-dependent and -independent. And like fibulin-3, IGFBP5 has been shown to play an oncogenic or tumor suppressive role depending on tissue cell context. In bladder cancer, IGFBP-5 overexpression was reported to be a poor prognostic factor in urothelial cancer patients (29), supporting the possibility of IGFBP5 as a tumor promoter and a pro-invasive factor alongside fibulin-3. Further proof of this was seen in our investigation when IGFBP5 overexpression increased the invasive and migratory potential that was lost in both of the fibulin-3 knockdowns in the T24 and UMUC-13 cells. Moreover, we also saw an increase with IGFBP5 expression when fibulin-3 was overexpressed in the 5637 and RT4 cells, suggesting a connection between these two genes. It

remains to be determined how fibulin-3 and IGFBP5 are exactly related to each other and how they function. One of the multifunctional roles of IGFBP5 involves interacting with the extracellular matrix to regulate cell attachment (30). In MCF-7 breast cancer cells, IGFBP5 increased cell adhesion through direct interaction with α2β1 integrins (31) that in turn enhanced cell survival, but inhibited cell migration. Conversely, IGFBP5 was found to enhance binding of IGF-1 to vitronectin, increasing cell migration in MCF-7 cells (32). These findings primarily looked at migration and not necessarily the invasion of cells, but it may be possible that IGFBP5's ability to promote or inhibit migration and even invasion depends on their binding partners and microenvironment (i.e. extracellular matrix). Since fibulin-3 is an extracellular matrix glycoprotein and IGFBP5 has been found to bind and interact with components of the extracellular matrix, it is plausible that IGFBP5 and fibulin-3 interact in a fashion that enhances cell adhesion in its given microenvironment to modulate this pro-invasive behavior, which may or may not be bladder cancer specific. Nevertheless, further studies need to be performed to delineate how fibulin-3 and IGFBP5 interact to regulate invasion.

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# Chapter V

## Discussion and Future Directions

In summary, using patient-derived tissue and *in silico* confirmation, our study demonstrated that fibulin-3 expression is upregulated in MIBC and is involved in promoting invasion and migration of bladder cancer cells *in vitro* and muscle invasive bladder tumors *in vivo*. Fibulin-3 induced IGFBP5 expression, which in turn promoted bladder cancer invasion. This has been the first time an association has been made between IGFBP5 and fibulin-3 and their roles as pro-invasive factors. Thus, fibulin-3 and IGFBP5 may serve as potential biomarkers and therapeutic targets for MIBC.

Although the results were not shown, numerous attempts were made to discover mechanisms through which fibulin-3 mediated its pro-invasive ability. Prior to identifying IGFBP5's involvement, we had extensively searched scientific literature to identify clues as to how fibulin-3 mediates this invasive phenotype. As mentioned earlier, we had examined MMP expression with gelatin zymography and MMP ELISAs, but did not find significant differences in MMP expression amongst the transduced cell lines. Due to fibulin-3's reported ability to activate signaling mechanisms, such as MAPK and AKT, we initially looked into signaling pathway reporter assays, specifically the phospho-kinase array. There were not significant differences seen with the fibulin-3 knockdown compared to their scrambled controls. EGFR and EMT were also examined with these transduced cells, which did not show significant changes as well. These attempts were followed by gene microarray with the Affymetrix Human Gene ST 2.1

plates. While revealing differences in gene expression, the differential expression was not validated upon qRT-PCR. All in all, these experiments suggest that fibulin-3's pro-invasiveness is not related to its ability to activate signaling pathways nor EMT. In addition, it reveals that manipulation of fibulin-3 expression does not result in large gene expression changes.

In addition, this animal model did not permit us to evaluate NMIBC due to the thin layer of lamina propria in mice. On a related note, it would be of interest to see if the manipulation of fibulin-3 expression in transgenic mice could inhibit/promote muscle invasive bladder tumors in the context of environmental induction of bladder cancer, such as BBN exposure. This would also allow us to explore both the environmental and genetic factors involved in the development of bladder cancer.

Further studies with fibulin-3 and IGFBP5 are also of interest, particularly whether they interact with each other to regulate the extracellular matrix to mediate their pro-invasive property. Although we saw both (1) invasion restored with IGFBP5 overexpression and (2) that the overexpression of fibulin-3 in the 5637 and RT4 cells resulted in increased IGFBP5 expression, we can only speculate IGFBP5's involvement with fibulin-3 in promoting the invasion and migration of these cells. Further manipulation of IGFBP5 expression in these cells would allow us to observe any differences in invasion/migration. Determining IGFBP5's direct involvement with fibulin-3 in invasion would allow us to conclude both fibulin-3 and IGFBP5's contribution to their pro-invasive feature.

Since both fibulin-3 and IGFBP5 are secreted proteins, it would be of interest to see whether these proteins could be detected in urine and whether they would show differences between NMIBC vs. MIBC, exploring the possibility of these genes as biomarkers. In addition,

future studies in the development of drugs that could therapeutically target fibulin-3 and/or IGFBP5 specifically in the bladder of MIBC would be of relevance.

Lastly, when we had validated the four candidate genes identified from RNA-Seq, we had interestingly found DUSP2 to show the opposite expression pattern as fibulin-3, showing higher protein expression in RT4 and 5637 and low expression in T24 and UMUC-13 cells. Although it was not thoroughly investigated in this study, it would suggest the possibility of DUSP2 expression also being involved in regulating bladder cancer invasion with DUSP2 expression being inversely correlated with invasion and migration in contrast to fibulin-3 expression. In fact, our preliminary studies with DUSP2 had verified that DUSP2 expression was lower in the MIBC vs. NMIBC as well as an attenuation of invasion upon DUSP2 overexpression in T24 and UMUC-13 cells. Thus, further investigation of DUSP2 and its role in bladder cancer could be of interest and it would be curious to see if it has any connections with fibulin-3.

In conclusion, due to its potential for lethal progression and the cost to society for prolonged monitoring, bladder cancer is a major burden to society. Thus, it is critical that an improved understanding of the mechanism of bladder cancer invasion be identified. Towards that end, we have demonstrated a novel mechanism that contributes to muscle invasion of bladder cancer. Specifically, we identified fibulin-3 as a pro-invasive factor involved in MIBC. Furthermore, we demonstrated that fibulin-3 mediates invasion, in part, through downregulation of IGFBP5. These findings provide the rationale to explore fibulin-3 as a clinical biomarker and therapeutic target for bladder cancer.